Testing changes in gene expression profiles in Octopus vulgaris (Mollusca Cephalopoda)

Thesis

TESTING CHANGES IN GENE EXPRESSION PROFILES IN *OCTOPUS VULGARIS* (MOLLUSCA CEPHALOPODA)

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Ilaria Zarrella

Laurea in Biotecnologie indirizzo industriale
Università degli Studi di Napoli Federico II

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Naples – Italy

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Director of studies: Dr Graziano Fiorito
Laboratory of Functional and Evolutionary Ecology
Stazione Zoologica A. Dohrn
Naples, Italy

Internal supervisor: Prof Roberto Di Lauro
Stazione Zoologica A. Dohrn
Naples, Italy

External supervisor: Prof Emiliana Borrelli
Microbiology & Molecular Genetics
University of California
Irvine, CA

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ABSTRACT

The aim of this thesis is to contribute to the understanding of the molecular machinery involved in learning and memory processes in *Octopus vulgaris*. Fear is the ***leitmotif***. A fear conditioning training protocol was developed to evaluate behavioural responses in animals negatively conditioned to an artificial stimulus. To test whether interaction with conspecifics in a solitary animal induces a form of innate fear, experiments were carried out to test the influence of 'social' interaction on predatory performances.

Genomic information available for *O. vulgaris* is limited, from these data I found *α-tubulin*, *octopressin*, *cephalotocin*, *stathmin*. I also identified the partial cDNA sequences for *TH*, *uch* and *dat*. *Creb* and *ubi* were also considered herein.

I studied the pattern of distribution of these genes by *in situ* hybridization, the analysis of the co-localization of *Ov-TH* and *Ov-dat* transcripts allowed to draw a possible distribution of dopaminergic and noradrenergic neurons in the octopus CNS.

I analysed the pattern of expression of these genes in response to fear. I showed that CREB phosphorylation levels significantly increased during memory retrieval suggesting that a phenomenon analogue to reconsolidation may occur in octopus.

Experiments of qRT-PCR revealed the increased expression of *Ov-uch* and *Ov-stm* in the lobes known as centers for learning and memory confirming the involvement of these genes in the processes of synaptic plasticity, learning and LTM.

The increased expression of *Ov-dat* and *Ov-TH* in response to learned fear suggests that the consolidation of a task with aversive reinforcers is mediated by a dopaminergic pathway. On the contrary, in response to social interaction these genes are down-regulated suggesting that this process is mediated by other neurotransmitters.

Finally, this study will provide the basic tools for future experiments where the analysis of the molecular machinery may be correlated with different forms of learning and synaptic plasticity.

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-	negative reinforcement
+	positive reinforcement
°C	degree Celsius
5-HT	serotonin
A. aegypti	Aedes aegypti
Ab	antibody
A.Ba. or ABL	Anterior basal lobe
Ab-Actin	antibody anti-Actin
Abbr.	abbreviation
Ab-CREB	antibody anti-CREB
Ab-pCREB	antibody anti-phosphorylated CREB
AC	Alternating Current
A. californica	Aplysia californica
A. Ch. or ACL	Anterior chromatiphore lobe
ACT	Aspartate kinase-Chorismate mutase-TyrA
A. mellifera	Apis mellifera
ANOVA	Analysis of Variance
AP	Alkaline phosphatase
APL	Anterior pedal lobe
Ap-uch	Aplysia ubiquitin carboxyl terminal hydrolase
Arg	Arginine
asic-1	acid-sensing/Amilioride sensitive ion channel family
AT	Attack
AVP	Vasopressin
BAL	Basal lobe
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BH2	dihydrobiopterin
BH4	tetrahydrobiopterin
Bis-Tris	Bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane
BL	Buccal lobe
Blast	Basic Local Alignment Search Tool
B. mori	Bombyx mori
bp	base pair
Brach or BRL	Brachial lobe
BSA	Bovine Serum Albumin
Bucc	Buccal lobe
bZIP	basic Leucine Zipper Domain

C	Cranium
C/EBP	CCAAT/Enhancer Binding Protein
CaMK	Calcium/Calmodulin dependent kinase
cAMP	cyclic Adenosine MonoPhosphate
casy-1	CAYSINtenin/Alcadein homolog
cat-2	abnormal CATecholamine distribution
CBP	CREB binding domain
cDNA	Complementary Deoxyribonucleic Acid
CEE	Comunità Economica Europea
C. elegans	Caenorhabditis elegans
c-Fos	cellular-Fos
CG	cerebral ganglia
CGRP	calcitonin gene-related peptide
c-Jun	cellular-ju-nana
CL	Ceiling Latency
Cl	chloride
cm	centimeter
c-Myc	cellular-myelocytometosis
CNS	Central Nervous System
C. quinquefasciatus	Culex quinquefasciatus
CR	cortical region
CRE	cAMP responsive element
CREB or creb	c-AMP Responsive Element Binding protein
CRF	corticotrophin releasing-factor
crit	criterion
CS	Conditioned Stimulus
Ct	cycle threshold
CT	Cephalotocin
CT	connective tissue
CTA	conditioned taste avoidance
CTR	Cephalotocin Receptor
Cy	cyanine
DA	dopamine
DAT or dat	Dopamine Transporter
D.Ba	Dorsal basal lobe
DBH	Dopamine β-Hydroxylase
dbxref	database cross-reference
DD	Dopamine Deficient
DEPC	Diethyl Pyrocarbonate
df	degrees of freedom
Dig	digoxigenin
DL	Decreto Legge
D. melanogaster	Drosophila melanogaster
DML	Dorsal Mantle Lenght
DNA	Deoxyribonucleic Acid

Dnase	deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
DOPA	3,4-dihydroxyphenylalanine
dsDNA	Double-Stranded DNA
DSP4	N-Ethyl-N-(2-chloroethyl)-2-bromobenzylamine hydrochloride
D-stathmin	Drosophila stathmin
dT	deoxythymidine
DTT	Dithiothreitol
EDD	Evolution, development, diversity
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular signal- Regulated Kinase
EST	Expressed Sequence Tagged
eu_TyrOH	eukaryotic tyrosine hydroxylase domain
F	Forward
FC	Fear conditioning
Fig.	Figure
Flu	fluorescein
fmn	fumin
g	gyrus
g	gramme
Gcn4	General Control Nondepressible
GenPept	Gene Bank Gene Products
Gln	Glutamine
glr-1	Glutamate Receptor family
Glu	Glutamic acid
GnRH	gonadotropin-releasing hormone
GO	Gene ontology
h	hour
H2O	Dihydrogen Monoxide
H2O2	Hydrogen peroxide
H3	Histone 3
HCl	Hydrochloride
hen-1	HesitatioN behaviour
His	Histidine
H.magnipapollata	Hydra magnipapollata
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
HW	dorsal Head Width
I. Fr. or IFL	Inferior Frontal lobe
ID	Identification number
IgG	Immunoglobulin G
ITI	inter-trial interval
J	Jelly
JNK	c-Jun N-terminal Kinase

Kb	Kilobase
KCl	Potassium Chloride
KD	knockdown
KDa	kilodalton
KID	Kinase Indicible Domain
KO	knockout
l	liter
LA	Latency to Attack
LB	Lysogeny broth or Luria-Bertani broth
LBD	ligand binding domain
L-DOPA	3,4 – dihydroxy-L-phenylalanine
Leu	Leucine
L. stagnalis	Lymnea stagnalis
LTF	Long Term Facilitation
LTM	Long Term Memory
LTP	Long Term Potentiation
Lys	Lysine
M	molar
M. Ba.	Medial basal lobe
magi-1	Membrane Associated Guanylate Kinase Inverted
MANOVA	Multivariate Analysis of Variance
MAPK	mitogen-activated protein kinase
mg	milligram
MgCl2	Magnesium Cloride
MgSO4	Magnesium Sulphate
min	minute
ml	milliliter
ML	Mantle Length
mm	millimeter
mM	millimolar
M. musculus	Mus musculus
mPFC	medial pre-frontal cortex
mRNA	messenger RNA
MSF	Medial Superior Frontal Lobe
MTM	Medium-Term Memory
MW	dorsal Mantle Width
N	number
N	neuropil
n.a.	not available
Na	sodium
NA	Noradrenaline
NA	No Attack
NaCl	sodium cloride
NBT	nitroblue tetrazolium
NCBI	National Center for Biotechnology Information

ncRNA	not coding protein RNA
NET	norepinephrine transporter
NF-kB	Nuclear Factor kappa B
ng	nanogram
nm	nanometer
NO	Nitric Oxide
NP40	nonyl phenoxylpolyethoxylethanol
npCREB	not phosphorylated CREB
NS	Not Significant
NTE	NaCl-Tris-EDTA
O	oesophagus
O. bimaculoides	Octopus bimaculoides
O. c. or OC	Optic commissure
O.C.T.	optimum cutting temperature
O. G.	Optic gland
oct-GnRH	octopus gonadotropin-releasing hormone
oct-GnRHR	octopus gonadotropin-releasing hormone receptor
OL	optic lobe
Olf. or OLF	Olfactory lobe
OP	Octopressin
Op18	Oncoprotein 18
OPR	Octopressin Receptor
OT	optic tract
OT	oxytocin
Ov-creb or Ov-CREB	Octopu vulgaris creb
Ov-dat or Ov-DAT	Octopus vulgaris dopamine transporter
Ov-stm	Octopus vulgaris stathmin
Ov-TH	Octopus vulgaris Tyrosine hydroxylase
Ov-tubA	Octopus vulgaris alpha tubulin
Ov-ub/S27A or Ov-ubi	Octopus vulgaris ubiquitin subunit 27A
Ov-uch or Ov-UCH	Octopu vulgaris ubiquitin C-terminal hydrolase
O. vulgaris	Octopus vulgaris
P or p	probability
pAvoid	percentage of avoidance
PBAL	Posterior basal lobe
PBL	Posterior buccal lobe
PBS	Phosphate Buffered Saline
PBRL	Pre-brachial lobe
PCL	Posterior chromatophore lobe
PCR	Polymerase chain reaction
pCREB	phosphorylated CREB
PDB	Protein Data Bank
PFA	paraformaldehyde
pg	picogram

pH	Potential of Hydrogen
PhD	Doctor of Philosophy
Phe	Phenylalanine
PKA	Protein Kinase A
PKC	Protein Kinase C
pmol	picomole
PMSF	Phenylmethanesulfonyl Fluoride
PNMT	Phenylethanolamine N-methyltransferase
PPL	Posterior pedal lobe
Pro	Proline
PSD	Protein Sequence Database
PVL	Palliovisceral lobe
R	Reverse
R	regulatory subunit
R	Red ball
Ref	references
RefSeq	Reference Sequence
RNA	Ribonucleic Acid
Rnase	Ribonuclease
R. norvegicus	Rattus norvegicus
rpm	rotations per minute
rRNA	ribosomal RNA
RT	Reverse Transcription
rt	room temperature
RT qPCR	Real-time quantitative PCR
sec	second
S. Fr.	Superior frontal lobe
SCG10	Superior Cervical Ganglia 10
SCLIP	SCG like-protein
SDS	Sodium dodecyl sulfate
SEM	supraoesophageal mass
Ser	Serine
SERT	Serotonin transporter
S.Fr. or SFL	Superior frontal lobe
SLD	Stathmin Like Domain
SLFL	Superior lateral frontal lobe
SOC	Super Optimal Broth
SSC	Standard Saline Citrate
STF	Short Term Facilitation
STM	Short Term Memory
stm	stathmin
SUB	Suboesophageal mass
Sub.Fr. or SUBFL	Sub-frontal lobe
SubV. Or SVL	Sub-vertical lobe
SWIP	Swimming-Induced Paralysis

Swiss-Prot	Swiss Protein Database
T	transparent ball
T. guttata	Taeniopygia guttata
TAE	Tris-Acetate EDTA buffer
TAM	tamoxifen
tetO	tetracycline Operator
TH	Tyrosine Hydroxylase
Thr	Threonine
TMD	Trans-Membrane Domain
TNB	Tris-NaCl-blocking reagent
TNT	Tris-NaCl-Tween
TrEMBL	Translated EMBL
Tris	Tris(Hydroxymethyl)aminomethane
Tris-HCl	Tris base and Hydrochloric acid
tRNA	Transfer Ribonucleic Acid
TSA	Tyramide Signal Amplification
TT	octopuses subjected to training and testing phases
tTA	tetracycline resonsive tran-activator
TTBS	Tween TBS
Tyr	Tyrosine
tubA	alpha tubulin
U	unit
UBP	ubiquitin-specific processing protease
UCH or uch	ubiquitin carboxyl terminal hydrolase
Uchl or UCH-L	ubiquitin carboxyl-terminal esterase L1
UK	United Kingdom
UniProt	Universal Protein Resource
US	Unconditioned Stimulus
UV	Ultraviolet
V	Volt
v	volume
V. M. or VAL	Vasomotor lobe
VL or V	Vertical lobe
VML	Ventral Mantle Lenght
vs	versus
w	weight
W+	white ball with positive reinforcement
WB Gene ID	identification number of the gene in the Wormbase
µg	microgram
µl	microliter
µm	micrometer

PREFACE

This thesis is the final work of my PhD study conducted from November 2003 to September 2011 at the Department of Functional and Evolutionary Ecology of the Stazione Zoologica Anton Dohrn in Naples. The subject of my work has been the identification and study of target gene (*Ov-dat, Ov-stm, Ov-TH, Ov-uch, Ov-CT, Ov-OT*) expression and protein activation (Ov-CREB) in octopus brain in response to learned and innate fear.

This dissertation consists of ten chapters. The first chapter is a general introduction to the topic of the thesis and it is divided in three major sections: first, a short review of studies on behaviour, learning and memory in some model animals, then a description of the model system used for this study, *Octopus vulgaris,* finally there is the description of aim and strategy of this project. Each chapter from 2 to 8 contain a brief introduction, a section dedicated to materials and methods, the analysis of results and a discussion. Chapters 2 describes the behavioural studies conducted on octopuses to analyze learned and innate fear. Chapter 3 and 4 are focused on knowledge of cephalopod and octopus gene sequences to find the target genes for this study. Chapter 3 summarizes the state of art of knowledge of cephalopod gene sequences, while in the chapter 4 are showed the strategies utilized to identify some octopus cDNA sequences unknown before. In the chapter 5 are reported the results of spatial expression studies of target genes in the octopus brain. Chapter 6 is dedicated to the study of role played by Ov-CREB phosphorylation in learned fear. Chapter 7 and 8 contain the studies of target gene expression (using RT qPCR) respectively in response to learned and innate fear, while the chapter 9 contains the comparison between two studies. The chapter 10 summarises and discusses the results obtained and some future perspectives are presented.

CHAPTER 1

INTRODUCTION

1.1 Behaviour, learning and memory in invertebrates.

How the brain generates functional output, ranging from locomotion, decision-making and learning and memory, remains poorly understood. Certainly the knowledge of these processes would help to better interpret the behaviour of animals, their ability to respond and adapt to environmental changes, to interact with each other and then more generally improve the knowledge of ecology. Obviously this knowledge, by moving from simple to more complex vertebrate animal models, could lead to an understanding of the mechanisms underlying complex human behaviours also influenced by emotions (sociality, altruism, emphaty, frustration, motivation, hatred, jealousy, alienation, racism, fear). This will surely help to explain, interpret and probably solve the problems underlying neuropsychiatric and neurodegenerative disorders that cause interference with behaviour of man with his capacity for learning and memory.

In recent years, molecular genetics together with behavioural analyses on model organisms have helped to identify genes involved in the formation of neuronal circuits, the execution of behaviour and mechanisms involved in the complex processes of learning and memory (as reviewed in Kandel, 2001, Davis, 2005; De Bono, 2005; Hawkins *et al.*, 2006, Romano *et al.*, 2006; Vosshall and Stocker, 2007; Engel and Wu, 2009; Ardiel and Rankin, 2010). However the situation in the highly complex brains of vertebrates is often difficult to interpret.

Genetic analysis in relatively simple invertebrate organisms has the potential to provide important insights into the relationship between genes, neuronal circuits and behaviour and also provides the potential to gain insight into the processes underlying the molecular bases of some human disorders (as reviewed in Barco *et al.*, 2006; Davis, 2005; Farooqui, 2007). These behavioural genetics studies provide the information to understand how genes and regulatory sequences may contribute to the organization and functioning of neural circuits and molecular pathways in the brain that support some animal behavioural responses to several types of learning (table 1.1).

Table 1.1 - A tabularized overview of several types of learning studied in animal models.

Non-associative learning	
Dishabituation	generated when the animal is presented with a novel stimulus and a partial or complete restoration of a habituated response occurs
Gill- and siphon withdrawal reflex	is an involuntary, defensive reflex that causes the delicate siphon and gill to be retracted when the animal (*Aplysia*) is disturbed
Habituation	generated when a stimulus is repeatedly presented to an animal and there is a progressive decrease in response to that stimulus.
Sensitization	consists in the increase of the animals response at the presentation of a novel, often noxious, stimulus
Associative learning	
Classical conditioning	a learning process that occurs through associations between an environmental stimulus (unconditioned stimulus) and a naturally occurring stimulus (conditioned stimulus) that is able to generate a conditioned response
Operant conditioning	an association formed between a behaviour and a consequence for that behaviour. It is a method of learning that occurs through rewards and punishments for behaviour.
Other form of learning	
Imprinting	an animal rapidly learns during a particular critical period to recognize an object, individual, or location, it is retained indefinitely
Problem solving	a process involved in the solution of a problem, usually allows an animal to move from a given state to the desired goal state.
Spatial learning	a form of learning that regulates animal movements in the environment to obtain food (or reward), or to avoid predators (or negative reinforcement).
Social or observational learning	a type of learning that occurs as a function of observing, retaining and replicating novel behaviour executed by others, usually a conspecific.

But they also contribute to the idea that environmental changes can interact with information

in the genome to modulate brain activity. Genes do not directly affect behaviour, but rather they encode molecular products that build and govern the functioning of the brain through which behaviour is expressed. Brain development, brain activity, and behaviour all depend on both inherited and environmental influences, and there is increasing confirmation that the molecular mechanisms regulating these processes are conserved between organisms of different species and in many cases also between invertebrates and vertebrates (as reviewed in Kandel, 2001). This explains the significant use of invertebrate organisms as animal model for studies of neuroscience to interpret complex behavioural responses, learning and memory processes in animals with simple nervous system (for review see Kandel, 2001). Learning, that is what makes memory formation, is often defined as a change in animal behaviour in response to experience. The persistence of this behavioural change over time is memory. The memory formation process has three stages : acquisition, consolidation and retention (see table 1.2).

Table 1.2 A tabularized overview of the stages of memorization processes.

1. Acquisition	Involves the initial perception of new experience
2. Consolidation	It is the process that allow the stabilization of memory trace after the initial acquisition.
3. Retention	
3.1 Reconsolidation	It is the process that allows to recall and actively consolidate the previously consolidated memory. Once memories undergo the process of consolidation and they become stable. However, the retrieval of a memory trace can cause another labile phase that then requires an active process to make it stable after retrieval is complete.
3.2 Extinction	It is evoked by the presentation of stimulus or context that triggers contextual memory retrieval. Memory extinction is a process in which a conditioned response gradually diminishes over time as an animal learns to uncouple a response from a stimulus

The first stage (acquisition) is more strongly related to the learning phase, when the animal is faced with the new experience of training. The consolidation stage is the phase when the memory is forming and stabilizing. The last stage (retention) refers to the recall of memory stored. During the retention phase the animal has to be re-exposed to training conditions, on the base of re-exposure duration to the learning context the memory course switch towards reconsolidation or extension (i.e. Pedreira and Maldonado, 2003). Alternatively, if reconsolidation does not occur the process of extinction will lead to 'loss' of the memory. Modern studies in cognitive neuroscience have shown that memory is not a unitary process but consists of several forms that can be grouped into at least two general categories each

with their own rules (Polster *et al.*, 1991; Squire and Zola-Morgan, 1991). Explicit, or declarative, memory is the conscious recall of knowledge of facts and events about people, places, and things, and it is a concept principally developed to explain the cognitive processes in the vertebrate brain. Implicit, or nondeclarative, memory is memory for motor skills (i.e. riding a bike) and other tasks (i.e. playing a musical instrument) and is expressed through performance, without conscious recall of past experience; it includes simple associative forms, such as classical conditioning, and nonassociative forms, such as sensitization and habituation (table 1.1). For both implicit and explicit memory diverse forms of memory storage have been described in several animal model which are summarized in the table 1.3 (De Zazzo and Tully, 1995; Kandel, 2001; Steidl *et al.*, 2003; Tomsic *et al.*, 2009).

Table 1.3 Schematic overview of the terms utilizedd to describe different forms of memory storage.

Short-term memory	which occurs immediately after training (that is, learning), but which decays within minutes, it is a transient and labile form of memory
Medium- or intermediate-term memory	which develops within minutes and lasts several hours, it is a labile form of memory
Anaesthesia resistant memory	which develops within an hour and lasts several days
Long-term memory	which requires repetitive training, and which develops several hours after training and lasts for more than a week. Long-lasting memory is usually defined as that which lasts at least one day and which is resistant to various forms of disruption, such as electroconvulsive shock or anaesthesia. It depends on protein-synthesis.

The temporal distinction and duration of each memory phase depends on the number of training trials and inter-trial intervals (ITI). The study of many species have shown that when training involves multiple trials, the time interval between trials is an important variable in the efficacy of accumulating training effects and strength of retention. Usually massed training characterized by short ITIs favours the formation of shorter lasting memory in respect to spaced training (i.e. Beck and Rankin, 1997; Botzer *et al.*, 1998; Menzel *et al.* 2001). Moreover the several forms of memory are reflected in specific forms of synaptic plasticity as well as in specific molecular requirements.

The short-term forms (STM) involve the covalent modifications of pre-existing proteins by a variety of kinases and are expressed as alterations in the effectiveness of pre-existing synaptic connections. By contrast long-term memory requires CREB-mediated gene expression and new mRNA and protein synthesis. Moreover, the long-term memory (LTM) requires new

synaptic connections. In addition to these two forms of memory (STM, LTM) a family of intermediate processes often require translation but not transcription can be produced by various training protocols using repeated or prolonged stimulation. The characteristics of these several forms of memory are conserved between vertebrate and invertebrate organisms (Kandel, 2001; Barco *et al.*, 2006). Below I will briefly summarize some of the most important discoveries about the role played by certain genes and molecular pathways in the regulation of behaviour, learning and memory of several key invertebrate species: *Aplysia, Caenorhabditis, Chasmagnathus, Drosophila.*

1.1.1 *Aplysia*

The marine mollusk *Aplysia californica* is one of key model organisms for studying the cellular and molecular mechanisms underlying learning and memory (reviewed in Bailey *et al.*, 1996; Carew and Sahley, 1986; Kandel, 2001). There are several advantages of using *Aplysia* as model system for the studies of synaptic plasticity, learning and memory storage. *Aplysia* has a relatively simple nervous system consisting of only 2 x 10^4 neurones but it is capable of a variety of behaviours. Moreover it is important to consider the dimension of *Aplysia* neurons, which are among the largest somatic cells in the animal kingdom, enabling genetic manipulation, such as the microinjection of plasmid, RNA, or protein (Kaang, 1996). Thanks to these advantages it was possible to improve knowledge of the numerous cellular and molecular processes that control neural circuits.

One of the behavioural responses most studied in this animal model is the gill- and siphon withdrawal reflex (mentioned in Table 1 above). This reflex exhibits several forms of learning, including habituation, dishabituation, sensitization, and classical conditioning, that have many of the behavioural features of learning in mammals, suggesting that learning in *Aplysia* and mammals may share common mechanisms (for review see Hawkins *et al.*, 2006). The molecular mechanisms contributing to implicit memory storage have been most extensively studied for the gill and siphon-withdrawal reflex of *Aplysia* (for review see Kandel, 2001). In particular, the studies conducted on the short-term memory formed in response to short exposure to noxious or sensitizing stimulus (i.e. tail shock, pulse of serotonine) showed the involvement of the enzymes adenylyl cyclase, protein kinase A (PKA) and the enhanced release of the transmitter glutamate by the sensory neurons onto its follower cells. These processes are accompanied by an increase in excitability of the sensory neurons attributable to the depression of specific sets of potassium channels. In addition, the changes in cAMP and calcium levels regulate different kinase and phosphatase activities (for review see Kandel, 2001).

A longer exposure to stimuli recruits activation of protein kinase C (PKC), Ca^{2+}/calmodulin-

dependent protein kinase (CamKII).

When synaptic stimulation reaches a given threshold or is repeated a number of times, it favours the increase of cAMP and leads to longer-lasting forms of synaptic plasticity. At the molecular level, this more robust stimulation causes the catalytic subunit of PKA to recruit p42 MAPK, and both then move to the nucleus where they phosphorylate nuclear targets (i.e. CREB1), including other kinases that, in turn, can phosphorylate transcription factors (i.e.CCAATbox- enhanced binding protein (C/EBP) and activate early gene expression (i.e. ubiquitin hydrolase) required for the induction of long-term memory (as reviewed in: Kandel, 2001; Hawkins *et al.*, 2006).

Critical chromatin changes also occur during the formation of long-term memory and these changes are required for the stable maintenance of these memories. Histone tail acetylation favours DNA transcription and is associated with active *loci.* Moreover the regulation of synaptic protein synthesis may play a role in the control of synaptic strength and it is located in the pre-synaptic cells of invertebrates (Martin *et al.* 1997).

However it was not possible to conduct many other molecular analyses due to the lack of knowledge of information about the genome of this animal model, until a few years ago. In 2003 Moccia and coworkers characterized the gene expression profiles of the processes of *Aplysia* sensory neurons, demonstrating the usefulness of the study of gene expression for understanding the processes of synaptic plasticity. Other genome information come from the sequencing of the *Aplysia* whole mitochondrial genome (Knudsen *et al.*, 2006), but more interesting for the studies in neuroscience was the sequencing of cDNA libraries from the central nervous system. Moroz, Kandel, and colleagues (Moroz *et al.,* 2006) sequenced more than 200,000 ESTs, which represented over 65,000 nonredundant sequences from *A. californica* cDNA libraries derived from the whole central nervous system (CNS), individual ganglia, identified neurons and identified processes of determined neurons. These gene sequences informed evolutionary studies and provide a useful tool to understand unknown molecular mechanisms activated in response to some behavioural experiences in a certain neuronal cells.

In conclusion, the model organism *Aplysia* despite being one of the first animals in which behavioural, physiological, pharmacological and molecular studies have been conducted to know the neural mechanisms activated in response to learning and memory, for a long time was missing adequate information of the genome. These studies of the neuronal transcriptome ensure a quick and solid progress in knowledge and interpretation of the plastic response of the CNS and its individual components in response to simple behaviours such as the gill-withdrawal reflex, though classic mutation based approaches are still lacking. The studies of this organism in the past have enabled comparisons to be made with the processes that occur in vertebrates. However it should be noted that the responses of the CNS are based on rather

simple behaviours. More complex behaviours are not manifested by *Aplysia* and this rather limits the usefulness of the model in this respect .

1.1.2 *Caenorhabditis*

One of the most important objectives of modern neuroscience is to find a common thread between genes, proteins, neural circuits, behaviour and learning in a single animal model. A good candidate for this purpose is *Caenorhabditis elegans*. Although this organism is very simple, it has a wide behavioural repertoire that can be easily understood, quantified and linked to appropriate genetic mutants that can explain its genetic and molecular bases (a facility lacking in *Aplysia* as pointed out above). This is made possible thanks to numerous studies that describe the anatomy of the nervous system (White *et al.*, 1986), behaviour (as reviewed in De Bono and Maricq, 2005; Giles and Rankin, 2009) and the completely sequenced genome of this animal (Hillier *et al.*, 2005).

Furthermore, the development of genetic manipulation techniques and genome-wide RNA interference libraries has facilitated the analysis of the functions of individual candidate genes (Kamath and Ahringer, 2003). Because *C. elegans* is transparent it is also possible to localize the expression of individual genes *in vivo* in distinct neuronal districts using differential interference contrast and fluorescence microscopy.

The large number of tools available for *C. elegans* researchers has allowed the identification of genes and key molecules that regulate the simplest behavioural responses together with several forms of learning. For example, *C. elegans* have been collected from many different parts of the world (Hodgkin and Doniach 1997; table A 1.1 in Appendix 1). Differences between these strains provide an opportunity to investigate at a molecular level the basis for natural phenotypic variation regarding their predisposition to social or solitary life in particular conditions such as the presence of bacteria (table A 1.1 in Appendix 1).

In addition, many behavioural studies have focused on the sensory processing of environmental stimuli that can change the locomotor response. When the worms find food they slow down and this response is due to activation of dopaminergic neurons. Experiments with genetic mutants have allowed the identification of some key genes that mediate this behavioural response (table A 1.1 in Appendix 1; Chase *et al.*, 2004).

Learning may be simply conceived as a change in the normal behaviour of an animal induced by environmental changes. One of the simplest forms of learning is the habituation (see table 1.1). Worms clearly can adapt to odorants (Colbert & Bargmann, 1995), habituate to mechanical stimuli (Rankin *et al.*, 1990; Wicks and Rankin, 1995), and change their behaviour according to their feeding state (e.g. Hills *et al.*, 2004). The neurons of *C. elegans* involved in the synaptic processes have been identified (for review see Giles and Rankin,

2009) and subsequently the molecular mechanisms activated in individual neurons during processes of learning and memory were studied (Lee *et al.*, 1999; Rose *et al.*, 2002, for review see Giles and Rankin, 2009; table A 1.1 in Appendix 1). In addition, what emerges from studies on thermotaxis, olfaction, chemotaxis, and mechanosensation is that worms are capable of associative learning. In all these paradigms, animals make an association between food (US) and a second stimulus (CS), with training requiring several hours. Conversely, animals can make a negative association between aversive stimuli, typically lack of food or starvation, and an initially neutral or positive stimulus (e.g. Saeki *et al.*, 2001,Wen *et al.*, 1997). The researchers have identified only some key genes involved in the associative learning processes and in synaptic plasticity (table A 1.1 in Appendix 1).

In conclusion, the study of behaviour and learned behaviour in *C. elegans* has been aided by the wealth of analytical tools and structural information available to researchers in the field and has led to fundamental advances in understanding of neuronal function, the identification of gene products and of molecular mechanisms that contribute to synaptic and neuronal function. At present, knowledge of the genes activated in the processes of associative learning is rather limited as can be seen from the information given in table A 1.1 in Appendix 1. This could perhaps be linked to the fact that so far the only limits to worm -learning in the laboratory seems to be the creativity of researchers in designing assays to evaluate performance.

1.1.3 *Chasmagnathus*

Twenty years ago, the crab *Chasmagnathus* was one of first invertebrates used as a model to study the neurobiology of learning and memory (e.g. Brunner and Maldonado, 1988; Lozada *et al.*,1990; Romano *et al.*, 1990, 1991; Tomsic and Maldonado, 1990; Tomsic *et al.*, 1991). To study the cellular and molecular mechanisms underlying these processes a simple learning task involving habituation was used. The absence of genome information has not helped the researchers to make much progress in this field. Pharmacological and few molecular approaches have been used to identify some key molecules involved in the consolidation, reconsolidation and memory extinction (table 1.2) cAMP-dependent protein kinase (PKA), extracellular signal- regulated kinase (ERK), the nuclear factor kappa B (NF-kB) transcription factor and histone 3 (H3) have all been found to be involved in the consolidation of long-term memory.

A huge amount of evidence shows that the cAMP pathway in neural plasticity is related to memory formation (*e.g.*, Castelluci *et al.*, 1982; Frey *et al.*, 1993). It is noteworthy to report that studies on this species provided the first data showing that the manipulation of PKA activity by means of cAMP analogues affects memory formation (as reviewed in Romano

et al., 2006). In addition, two members of the family of mitogen-activated protein kinases (MAPKs): extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) have been studied. ERK, but not JNK, showed memory specific-activation in the central brain.

Moreover (Freudenthal *et al.*, 1998; Freudenthal and Romano, 2000) experimental work demonstrated the involvement of NF-kB not only during consolidation, but also in reconsolidation. In animals with fully consolidated memory, a brief re-exposure to the training context induced neuronal NF-kB activation and reconsolidation, while prolonged re-exposure induced NF-kB inhibition and memory extinction (Merlo and Romano, 2008).

The interaction between transcription factors and chromatin is regulated by means of histone acetylation–deacetylation. In such a way, stable changes in gene expression are achieved by this regulation, which could be an important mechanism in the consolidation process for the stability of long-term memory. Federman and coworkers (2009) provided evidence that histone 3 (H3) acetylation has a fundamental role in the consolidation process induced by a strong training session.

The results of studies of *Chasmagnathus* emphasize the close relationship that exists between the molecular machinery that regulates the processes of learning and memory in invertebrates and vertebrates (i.e. de la Fuente *et al.*, 2011). But probably the lack of knowledge of the genome of this animal model and lack of investment in this area restricts their use in behavioural genetics studies.

1.1.4 *Drosophila*

Drosophila melanogaster as an experimental organism has contributed very much to contemporary neurobiology. During the period from 1968 until to 2000 many researchers selected *Drosophila* as a model organism for the study of fundamental problems in biology and neurology. From 2000, a new era for *Drosophila* has maturated: "the genome era" (Adams *et al.*, 2000; Myers *et al.*, 2000; Rubin *et al.*, 2000; Celniker *et al.*, 2002) when the complete genomic sequence was published. The sequencing and annotation of the *Drosophila melanogaster* genome has provided a number of important contributions to knowledge of molecular mechanisms involved in several biological and behavioural responses of this animal model.

A principal aim of neuroscientist studies is to understand the mechanisms and the neural circuits guiding behaviour and memory. Several advances in genetic technology have allowed to manipulate gene expression and observing the consequences on animal behaviour, to study the neural circuit involvement and even the individual neuron activation (e.g. Wilson and Stevenson, 2003; Wang *et al.*, 2004; Yu *et al.*, 2004; Schroder-Lang 2007).

These findings have transformed *D. melanogaster* from a useful organism for gene discovery to an ideal model to understand neural circuit function in learning and memory.

The genetic and molecular basis of olfactory learning and memory has been studied in *Drosophila melanogaster* for many years. The researchers employ classical conditioning training in which they associate an odour conditioned stimulus (CS) with either a punitive shock or a rewarding sugar unconditioned stimulus (US; Tempel *et al.*, 1983; Tully and Quinn, 1985; Schwaerzel *et al.*, 2003). After this training the fly memory is observable (for some hours or days depending on the training protocol) an avoidance or attraction response to the reinforced odour.

Several experimental approaches have been used to identify the key molecules involved in learning and memory processes: fly mutants, tools to disrupt and stimulate the function of genetically identifiable neurons, tools and techniques to optically image and directly record neural activity (e.g. Wilson and Stevenson, 2003; Wang *et al.*, 2004; Yu *et al.* 2004; Schroder-Lang *et al.*, 2007). The genes identified as essential for learning and memory of olfactory-related tasks are shown in table 1.4.

Beyond these neurogenetic studies conducted on olfactory learning and memory, many other behaviours have been productively dissected with genetic and behavioural tools in *Drosophila*. An example is the study of pre-copulatory courtship behaviour in *Drosophila*. This analysis has identified the brain regions and the genes (table A 1.2 in Appendix 1) that govern this process (for review see Vosshall, 2007).

Locomotor behaviour is regulated by the environment, but mutagenesis studies have revealed that this process is under genetic control (Konopka and Benzer, 1971). The genes that are involved in controlling the biological clock are listed in table A 1.2 in Appendix 1 (for review see Vosshall, 2007).

Extensive molecular analysis has been conducted on chemosensory behaviour. The availability of the genome sequence of *Drosophila melanogaster* opened this system to rapid identification of the sequence of 62 odorant receptors and 68 taste receptors (Vosshall, 2007) and the complete map of their expression in the central nervous system.

Aggression is another form of behaviour influenced both by genetic and environmental factors. Using microarray analysis, has been identified genes with differing expression levels in the aggressive and neutral lines. These genes (table A 1.2 in Appendix 1) are candidates to regulate a complex social behaviour like aggression.

About a dozen behavioural models together with a variety of genetic techniques can be combined with standard physiological, pharmacological approaches to study habituation in *Drosophila*.

In many of these studies, genetic mutations have been made to change the function or expression of certain molecules known to be involved in the process of habituation (i.e.

components of second-messenger signal transduction pathways, ion channels, and mediators of synaptic transmission; table A 1.2 in Appendix 1). Some of those mutations were first identified in screens for learning defects (forward genetics), while others were studied because their products seemed suited to play a role in synaptic plasticity (reverse genetics) (for review see: Davis, 1996; Dubnau and Tully, 1998; Waddell and Quinn, 2001). Many genes identified as key molecules for habituation have also been examined for effects upon associative learning, they may provide a potential bridge for linking the physiological mechanisms of different behavioural paradigms.

As a model organism, *Drosophila melanogaster* has been especially useful for the genetic dissection of developmental and anatomical traits. The fact that many genes found in flies have structural or functional homologues in vertebrates, including humans, means that genetic discoveries in the fruit fly can contribute to our general understanding of evolutionarily conserved developmental and physiological processes. It is an exceptionally useful genetic model for the study of simple and complex behaviours, and its use as such has given rise to an important body of literature, in which common themes on the molecular, cellular and evolutionary underpinnings of behaviour can be found. Moreover the progress in the knowledge of complete genome sequence of eleven additional species (Vosshall, 2007) open the possibility of new neurogenetics and behavioural studies aiming at the knowledge of mechanisms of species recognition, food preferences, chemosensory reception specally for species which occupy overlapping niches.

However, despite the profound and wide anatomical, molecular and functional knowledge of nervous system and learning mechanisms of *Drosophila*, scientific results from the last decade of research have rejected the hypothesis of single learning mechanism in *Drosophila.* It has multiple and overlapping molecular mechanisms and anatomical structures that are critical for learning and memory in different environmental conditions (Zars, 2010).

If it is true that many neurotransmitters, key molecules and entire signal transduction pathways have emerged early in evolution and have been largely conserved, it is possible to assume that the basic core of memory is the same from annelids to humans and that the main differences between species are given by the emergent properties that arise as a consequence of increasing complexity.

The development of learning and memory models in different taxa and the use of non conventional animals in these studies are important contributions to the search for molecular mechanisms involved in learning and memory processes.

Each animal model presents particular advantages and disadvantages for the study of some aspect of the subject. Behavioural responses to a determined task is different in each species, thus assorted species must be used to extract the general principles that govern

the molecular basis of complex behaviour. Dissecting it into its components can help to identify root similarities across distantly related species allowing a comparative analysis of the learning and memory processes.

Octopus vulgaris in some ways can be considered a non conventional animal model, but the richness of its behavioural repertoire, the robustness of his learning ability, the deep and wide knowledge of its central nervous system (CNS) definitely make it a valuable animal model on which to conduct investigations on learning and memory mechanisms also for comparative and evolutionary studies.

1.2 *Octopus vulgaris*

1.2.1 Why *Octopus vulgaris*?

The cephalopod mollusk *Octopus vulgaris* is an ideal candidate for the analysis of the molecular and neuronal mechanisms underlying complex behaviour. The octopus is able to adapt quickly to captivity. It requires just a small tank with running sea-water and a small house made of two bricks to acclimatize and to recover prompt responses to stimuli (for review see Borrelli & Fiorito 2008). Octopuses are naturally curious animals reacting to events with highly stereotyped predatory behaviour that can be easily interpreted and quantified allowing analysis of inter-individual variations including those due to experimental interferences (Boycott and Young, 1955b; Sanders, 1975; Borrelli, 2007).
Finally, the octopus is able to recover quickly after massive brain surgery (Boycott and Young, 1955a; Young, 1971; Shomrat 2008). Besides these advantages there are obviously some disadvantages such as lack of knowledge of the genome sequence, a CNS that, although less complex than a vertebrate brain, is one of the largest and most complex among invertebrates, moreover the cells of CNS in some lobes are very small (< 5 μm; figure 1.1 and 1.2). Moreover there is a limited amount of literature on molecular studies conducted on this animal and here I set out to in-part remedy this lack of information.

1.2.2 *Octopus vulgaris* central nervous system and visual and tactile stimuli processing

Complexity of the cephalopod neural system can be accounted for several istances: *i* cephalopods possess brains larger in size, relatively to their bodies, than those of lower vertebrates (i.e. fishes and reptiles; Packard, 1972); *ii.* the central nervous system of octopus accounts for about 300 million neurons, a number that is hundreds of times higher than that of other invertebrate species (i.e. *Aplysia*, *Apis*); *iii.* their neuroanatamical organization (for review see Young, 1971; Budelmann, 1995; Williamson and Chrachri, 2004; for summary of anatomical description see Appendix 3). Ablation experiments carried out on octopus brain over several decades (from '50s and '60s) provide indication of complex connectivity and inter-relationship between different areas within the octopus nervous system (for review see Young, 1991; Borrelli and Fiorito, 2008)

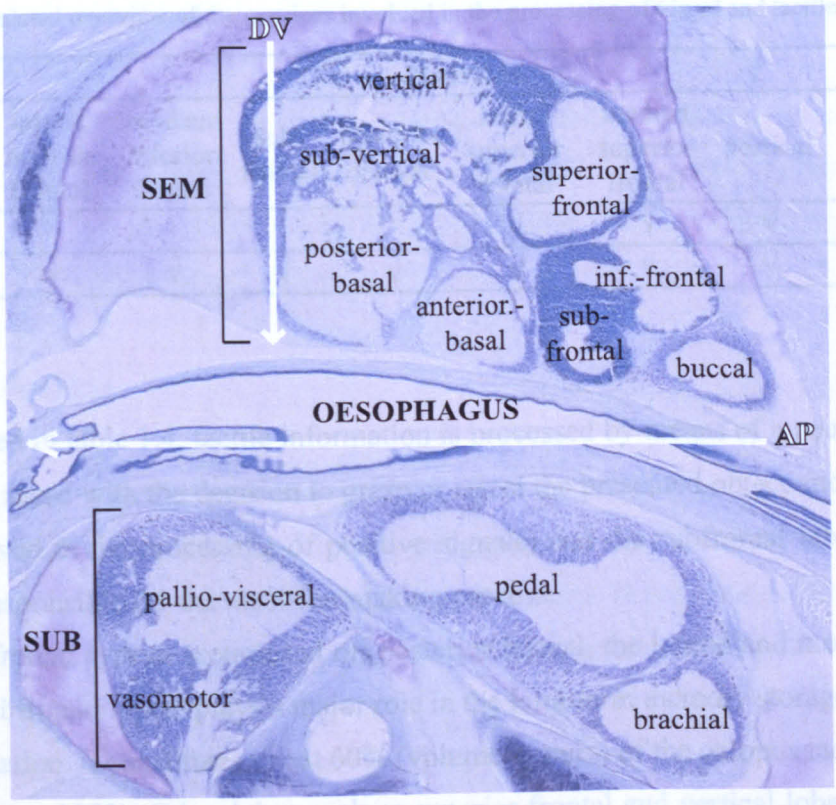

Figure 1.1: A sagittal section of the octopus brain after Nissl staining. Some of the lobes that constitutes supra-esophageal mass (SEM; above) and sub-esophageal mass (SUB, below) are identified (for details see Appendix 3).

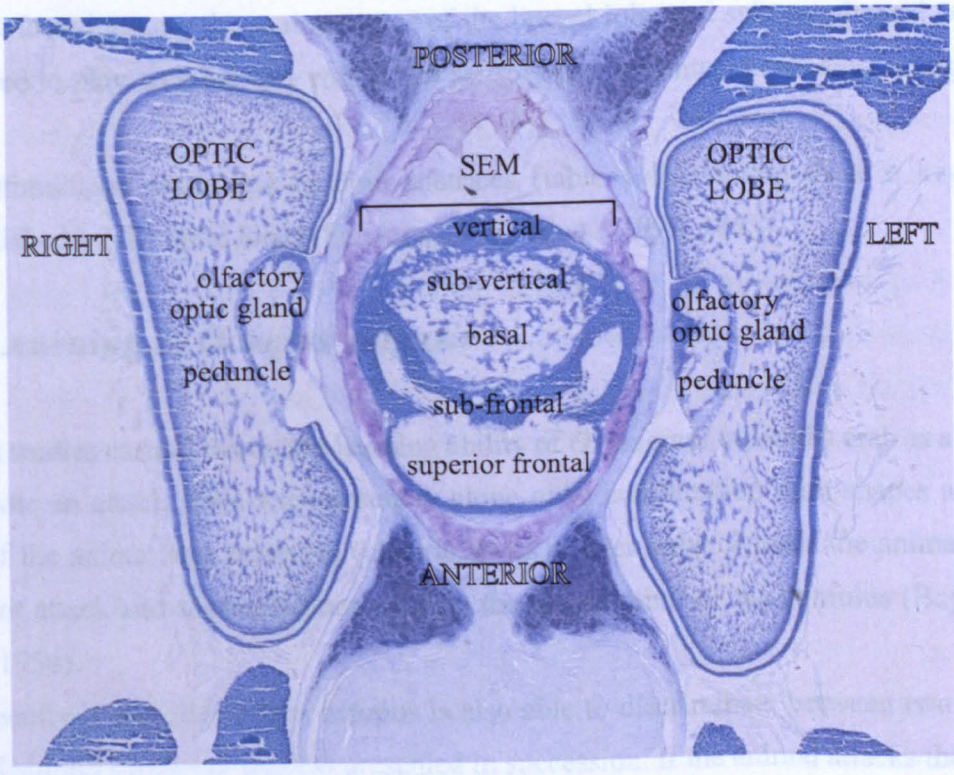

Figure 1.2: An horizontal section of the octopus brain after Nissl staining. Some of the lobes that constitutes supra-esophageal mass (SEM; in the center) and the two optic lobes (one for each side) are identified (for details see Appendix 3).

Table 1.4 - A tabulated overview of the matrices involved in the processing of visual and tactile stimuli.

	Matrices							
Stimuli	lateral inferior frontal	median inferior frontal	sub-frontal	posterior buccal	lateral superior frontal	median superior frontal	vertical	sub-vertical
visual					√	√	√	√
tactile	√	√	√	√	√	√	√	√

As schematized in table 1.4, tactile information is processed by means of a neural network, octopuses are faced with the decision to grasp or reject the presented object and the inferior frontal (involved in the processing of positive signals) and the subfrontal lobes (negative signals) are responsible for the decision making process.

The inferior frontal system (composed of: posterior buccal, the lateral and median inferior frontal and subfrontal lobes) plays a major role in the long-term memory storage process for tactile information. It constitutes about 60% (volume/system) of the octopus tactile learning capacity (Young, 1983). Other lobes such as superior frontal and vertical lobes (25%) and other parts of suboesophageal mass (15%; Young, 1983; for review see: Williamson and Chrachri, 2004) are involved in processing of tactile information. For example the amacrine cells distributed among the motoneurons of the buccal lobe and suboesophageal centre are considered to play an inhibitory role in the sensory-motor control to feeding/eating (Young 1991).

Visual stimuli are processed by four matrices (table 1.4). Among these a key role is accomplished by the optic lobes (Sanders, 1975; Young, 1991, 1995)

1.2.3 Learning in *Octopus vulgaris*

The first studies carried out on the learning ability of *O. vulgaris* utilized a crab as a stimulus to promote an attack. This was presented alone or in combination with shapes and every attack of the animal was punished (electric shock). After only 10 trials the animal learned the to not attack and responded correctly to the presentation of the stimulus (Boycott and Young, 1956).

Subsequently it was shown that octopus is also able to discriminate between two different artificial stimuli (different shapes) presented in succession. If the animal attacks the positive stimulus it is rewarded with a crab; when it attacks the negative stimulus a punishment (electric shock) is delivered. In successive experiments the crab was substituted by a small piece of anchovy to balance the requirements of repeated trials and overcome eventual motivation decline due to satiety (Young, 1961). The success of these initial behavioural

experiments positively influenced research in this area and documented the ability od octopus to learn different tasks.

Subsequently, sensitization, habituation, associative learning (avoidance, visual and tactile discrimination) and spatial learning were all shown to occur in *O. vulgaris* (review in: Young, 1961; Sanders, 1975; Wells, 1978; Boyle, 1986; Boal, 1996; Hanlon and Messenger, 1996; Hochner *et al.*, 2006; Borrelli and Fiorito 2008).

1.2.3.1 Sensitization

Following everyday practice with octopuses daily presentation of food (i.e. preys) increases the chance of the animal attacking. In this way its predatory performance improves over time, recovering what is expected to be its "individual attitude" that is eventually "disturbed" after capture from the wild. This is a clear case of sensitization (Wells ,1967; Chase and Wells, 1986; table 1.1).

1.2.3.2 Habituation

Classic studies have tested the capability of octopuses to habituate to take objects when unrewarded (Wells and Wells, 1956; table 1.1). In these studies, animals (usually blinded) require few trials to start to significantly limit the time spent exploring an object with its arms. Animals easily and realiably habituated to one object presented to them; this allows the same aniamls to be able to distinguish it from another one different in texture that is taken as soon as it is presented to the animal for the first time.

Recently, experiments have been carried out in the Fiorito laboratory at Stazione Zoologica. The octopuses are presented with a un-openable jar containing a live crab for at least 10 successive trials. During the initial trials the animal attacks the jar trying to capture the prey. During the training animals increase their latency to attack and reduce the time spent to explore the jar (Fiorito, unpublished).

1.2.3.3 Associative learning

A large number of protocols have been developed in octopus to study associative learning (table 1.1). Visual learning has been studied using the visual discrimination tasks. Artificial stimuli with different shapes (i.e. circle and rectangle, horizontal and vertical rectangle, L- and C-shapes) or different colour (i.e. white and black; red and white) have been presented simultaneously or successively to octopuses. They are reinforced with anchovy as reward (positive stimulus) or shock (negative stimulus). The octopuses are capable to discriminate

between the stimuli and learn to attack the positive stimulus and avoid the negative one (e.g. Boycott and Young 1956; Young 1956; Boycott and Young 1957; Sutherland 1958; Muntz *et al.*, 1962;).

Also tactile discrimination tasks have been utilized to study the octopus associative learning capabilities. Smooth or rough plastic spheres or cylinders have been used as stimuli (positive or negative) to induce the octopuses to discriminate through their tactile skills (e.g. Wells and Young 1965; 1969; Wells and Young 1970b; Wells and Young 1972, for review see: Sanders 1975).

Also many protocols of passive avoidance (table 3.1 in chapter 3) have been tested on octopus and a revised version of this protocol (see chapter 3; Borrelli, 2007; Shomrat *et al.*, 2008) has extended the "portfolio" of training paradigms that may be utilized to find answers to the fundamental question of how and to what extent *O. vulgaris* is capable of learning to modify its behavioural response.

1.2.3.4 Spatial learning

Spatial learning was originally tested in cephalopods using mazes; the question whether octopuses are capable or not of learning a detour has been debated (Schiller 1949; Boycott, 1954; Wells, 1967; but see Walker *et al.*, 1970; Moriyama and Gunji, 1997; table 1.1). During the last few decades, studies on the problem-solving abilities of these animals have been frequently confused and erroneously attributed to spatial learning processes (Fiorito *et al.*, 1990; but for critiques see Mather, 1995; Hanlon and Messenger, 1996). Nevertheless, spatial learning *sensu stricto* has now been shown in several species. Apart from the pioneering studies of Mather (1991) on *O. vulgaris*, learning to orient and navigate in space is reported for *O. bimaculoides* (Boal *et al.*, 2000) but a systematic analysis of spatial learning in *Octopus vulgaris* is, to the best of my knowledge, still missing.

1.2.3.5 Problem solving

Pieron described one of the earliest problem solving tasks in octopus. In 1911 he realized that the octopus is able to solve a problem solving task capturing a prey contained in a glass jar (table 1.1).

Fiorito and coworkers (1990, 1998) analyzed the ability of octopuses to open the jar and capture the prey. Octopuses initially guided by vision of the prey attack the jar and after physical contact with the jar are guided by their tactile ability to remove the plug to catch the prey. Performance times for both removing the plug and seizing the prey improve with experience on the task.

The response of animals that includes a switch between two modalities (from visual to tactile) is not automatic and for this reason it is different from animal to animal.

This is probably due to differences in the speed of each animal to recall and activate the motor programs that are needed to solve the task. In fact, for the octopus opening the jar and removing the plug includes the activation of a motor program that is already known and used by the animal to prey on a bivalve (McQuaid, 1994, Fiorito and Gherardi, 1999; Steer and Semmens, 2003) .

Also, catching prey in a jar (detour) without the aid of sight is a common technique used by the animal as a foraging strategy in nature guided by tactile skills octopuses go on a blind exploration of the sea-bottom searching for hidden prey (for review see: Hanlon and Messenger, 1996; Borrelli *et al.*, 2006). Recently new tasks (*e.g.* multi-openable jars, boxes with drawers) have been designed to further explore *O. vulgaris*' problem solving capabilities (Borrelli, 2007).

1.2.3.6 Social learning

The common octopus, notoriously considered a cryptic and solitary predator, is capable of observational learning (Fiorito and Scotto, 1992; table 1.1), it is able of utilizing information from conspecifics on how to solve a task. In brief, naïve octopuses are able to solve an unrewarded simultaneous visual discrimination task (a choice of white vs red plastic ball) after observing the performances of a previously trained conspecific.

1.2.4 Neurobiology of learning and memory

In contrast to a large number of papers published on the learning ability of *O. vulgaris* and the effect that lesions in some brain areas may induce on its behavioural performance (reviewed in Sanders 1975), very little is known about the ability of octopuses to recall information recorded during training (but see for example Boal, 1996). In addition, there are very few papers that report on the formation of short-term (STM), medium-term (MTM) and long-term memory (LTM) are reported (table 1.5).

What is really surprising is that in many cases what is commonly referred to as long-term memory lasts for a very long period (i.e. 'one month': Sutherland, 1957; 'some months: Sanders, 1970). However, despite the ability to remember for a long time, little is known about the time course of retention and consolidation activated in response to a specific behavioural experience, particularly in comparison to what is known in other model organisms (e.g. *Drosophila*, *Apis*). There are only a few examples that focus on the effect of administration of electroconvulsive shock on retention of memory after training (Maldonado 1968, 1969)

and of tetanization *in vivo* of the vertical lobe to study its consequences on learning, and short term and long-term memory (Shomrat *et. al.*, 2008).

Table 1.5 Different learning paradigms and training with different intervals (massed vs. spaced) used in *O. vulgaris* to produce memories of variable time span: hours (h), weeks (w). The extent of the recall at each stage (STM: short term memory; MTM: medium term memory; LTM: long term memory) has been measured by each of the cited papers. The references (Refs) included are: 1. Sutherland, 1957; 2. Maldonado, 1968; 3. Sanders, 1970; 4. Wells and Young, 1970a; 5. Sanders and Barlow, 1971.

Paradigm	Training	STM	MTM	LTM	Refs
Classical conditioning	Massed			48h	2
Classical conditioning	Massed			48h	4
Classical conditioning	Spaced			4w	1
Classical conditioning	Spaced			16w	3
Avoidance	Massed	1h	8h	24h	5

1.2.4.1 Electrophysiological approach

In the octopus central nervous system the key role for learning and memory processes is played by vertical lobe (VL; Sanders, 1975; Wells, 1978). Removal of VL impairs LTM and the ability to learn new tasks (e.g. classical conditioning Boycott and Young, 1955a; observational learning Fiorito and Chichery, 1995). Young (1995) suggests that the neurons of VL made their connection with those afferent from the medial superior frontal lobe (MSF) forming a matrix analogous to vertebrate hippocampus. This structural and functional similarity has been confirmed by electrophysiological studies conducted on a slice preparation from the VL system (Hochner, 2003). They found that the VL manifests long-term potentiation similar to that found in the vertebrate hippocampus, suggesting a convergent evolution of the synaptic processes activated during learning processes.

To study the role played by the tetanization of VL (obtained inducing global LTP *in vivo* by high-frequency stimulation of the MSF-VL system) and the connection of VL with MSF in the learning and memory processes some experiments of tetanization and MSF-VL transection have been conducted on octopuses subjected to a passive avoidance task (Shomrat *et al.*, 2008).

Both physiological and surgical approaches have caused changes in the function of VL that is a pivotal brain station for learning and memory systems. Thus, the input from MSF and VL, such as the output of MSF, is important but not crucial for short-term acquisition of the avoidance task. Our results support the previous ones which suggest that removal of VL or MSF or transection of MSF tract impairs but does not block the octopuses to avoid a crab during an associative learning task (Boycott and Young, 1955a; Maldonado, 1965).

Surprisingly the tetanized octopuses do not show any impairment in short term learning. During the testing phase we have checked for long-term memory of training experience. The transected and tetanized octopuses do not remember the training experience showing an impairment in log-term memory. In conclusion, LTP in the VL does not seem to be involved in the short-term learning process because tetanization does not impair learning but negatively affects long-term retention.

1.2.4.2 Pharmacological approach

Several pharmacological studies in octopuses have shown the effects of drugs on behavioural learning and memory indicating some plausible biochemical mechanisms for the cellular mediation of these processes. There are indications for a possible involvement of nitric oxide (NO) in both tactile and visual learning and memory processes in octopus (for tactile learning Robertson *et al.*, 1994, for visual learning Robertson *et al.*, 1996). NO is required for extension and bending of filopodia and these processes are essential for learning. These findings were confirmed by Robertson (1994) who studied the effect of cytochalasin D (an inhibitor of filopodial extension) on tactile learning process. Subsequently, Fiorito *et al.* (1998) suggested the involvement of acetylcholine in visual and tactile learning process activated in response to several behavioural tasks (e.g. problem solving, visual discrimination).

1.2.4.3 Molecular approach

Despite the large number of behavioural studies, there is little information on the molecular basis of *Octopus vulgaris* learning (as reviewed in Hochner *et al.*, 2006). This animal model suffers from insufficient and sometimes non-existent availability of tools for molecular studies that are widely available for other invertebrate organisms (such as *Aplysia, Caenorhabditis, Drosophila*). Some studies have been conducted on the distribution of neurotransmitters and neuromodulators in the *Octopus vulgaris* optic lobe and peduncle complex. In particular, immunohistochemical analysis has been used to investigate the distribution or colocalization of galanin and serotonin (Kito-Yamashita *et al.*, 1990; Suzuki *et al.*, 2000), neuropeptide Y and FMRFamide (Suzuki *et al.*, 2002b; Di Cosmo and Di Cristo, 1998), corticotrophin releasing-factor (CRF) and neuropeptide Y (Suzuki *et al.*, 2003), the distribution of calcitonin gene-related peptide (CGRP; Suzuki *et al.*, 2002a), gonadotropin-releasing hormone (GnRH; Di Cosmo and Di Cristo, 1998), acetylcholine (D'Este *et al.*, 2008) and glutamate receptors (Piscopo *et al.*, 2007).

Few other studies have been done on the distribution of molecules and receptors involved in learning and memory processes in octopus CNS. These studies usually include the analysis.

of the mRNA by *in situ* hybridization (e.g. octopressin and cephalotocin: Takuwa-Kuroda *et al.* 2003; oct-GnRH: Iwakoshi-Ukena *et al.*, 2004; oct-GnRH receptor: Kanda *et al.*, 2006; calretinin: Altobelli and Cimini, 2007) and /or protein distribution by immunohistochemistry experiments (e.g. NMDA receptor 2A and 2B: Di Cosmo *et al.*, 2004 and reviewed in Di Cosmo *et al.*, 2006; oct-GnRH: Iwakoshi-Ukena *et al.*, 2004; serotonin: Shomrat *et al.*, 2010).

Only one study has been published analyzing the distribution of transcripts of some target genes in the different masses of the octopus nervous system by RT qPCR (Sirakov *et al.*, 2009).

The promising results of these studies suggest expectations for future studies designed to identify molecular mechanisms involved in the complex processes of learning and memory.

1.3 Aims and strategy of this thesis

The aim of my PhD thesis is to contribute to the knowledge of the molecular mechanisms underlying learning and memory processes in the Cephalopod Mollusc *Octopus vulgaris,*. To achieve this goal I first focused on c-AMP responsive element binding protein (CREB), a transcription factor widely recognized as a key molecule in the formation of long term memory (LTM) in several organisms from invertebrates to vertebrates (Kandel, 2001). The large number of studies carried out to elucidate the biochemical mechanisms of memory formation and synaptic plasticity gave strong support to choose CREB as candidate molecule for these studies of memory. CREB activation is, in fact, recognized as a molecular switch that controls the transformation of a memory trace from labile and short to a long lasting form in both invertebrate and vertebrate organisms (e.g. Silva *et al.*, 1998; Tully *et al.*, 2003).

In the second part of my project, I analyzed the expression pattern of a set of genes in response to fear conditioning and social interaction in the octopus. Although preliminary, my results are the first exploration in the molecular mechanisms involved in fear conditioning (either instrumental and innate) in the same animal, the octopus. This is of particular interest due to the fact that *O. vulgaris* is a solitary living species (but see Anderson *et al.*, 2010; Tricarico *et al.*, 2011).

In order to explore any relationship between gene expression and learning in octopus, I utilized a biased approach. This was due: *i.* the limited available knowledge on *O. vulgaris* transcriptome, *ii.* a scanty representatives of nucleotide/protein sequences found in GenBank, *iii.* the noverlty of studies of this kind for the animal model (i.e. Cephalopod).

Ubiquitin hydrolase, stathmin, tyrosine hydroxylase, dopamine transporter, cephalotocin and octopressin were chosen as target genes for this project.

Ubiquitin C-terminal hydrolase is an immediate early gene that promotes proteasomal degradation by recycling ubiquitin from the poly-ubiquitin chains of degraded proteins (e.g. Hegde *et al.*, 1997). In this way the regulatory subunit of the protein kinase A (PKA) is degraded resulting in a persistent activation of PKA, thus converting a short-term signal into long-lasting synaptic changes as occurs in long-term facilitation in *Aplysia* (e.g. Hegde *et al.*, 1997) and in vertebrates (e.g. Jiang *et al.*, 1998).

Stathmin is a neuronal growth-associated protein that controls the microtubule stability and dynamics, an important factor of synapse development and plasticity (e.g. Ruiz-Canada *et al.*, 2004). In addition, stathmin knockout mice show deficits in spike-timing-dependent long-term potentiation (LTP) and impairment in memory recall of conditioned fear response (e.g. Shumyatsky *et al.*, 2005), thus suggesting that stathmin plays a role in the cellular context that underly neural plasticity including learning.

The other two genes chosen as target for this study were tyrosine hydroxylase (TH) and dopamine transporter (dat). TH is the rate-limiting enzyme of dopamine biosynthesis that participates in the control of intra- and extra-cellular levels of dopamine (e.g. Jones *et al.*, 1998; Jaber *et al.*, 1999). Dat clears neurotransmitters from the extra-cellular space and serves as an important regulator of signal amplitude and duration at dopaminergic synapses (Mortensen and Amara, 2003). Growing attention has recently been dedicated to the analysis of the role of TH and dat due the importance of dopamine modulation in various cognitive and behavioural processes in both vertebrates and invertebrates (e.g. Kobayashi and Kobayashi, 2001; Sanyal *et al.*, 2004; Zhang *et al.*, 2008; reviewed in Russell, 2007).

Octopressin and cephalotocin are homologs of vasopressin (AVP) and oxytocin (OT), which are members of a large group of ancient neuropeptides that have profound effects on a variety of mnemonic and social processes. AVP and OT have been shown to influence a number of forms of social behavior, in particular vasopressin influences courtship, affiliation and aggression in a wide range of taxa (e.g. Bardou *et al.* 2009; 2010; for review see Keverne, 2004; Winslow, 2004), whereas oxytocin takes a more dominant role in social interaction and recognition (reviewed in Sanchez-Andrade, 2009).

In order to evaluate changes, if any, in the activation of CREB and in the expression of ubiquitin hydrolase, stathmin, tyrosine hydroxylase and dopamine transporter, fear conditioning was the *leit motif* of this work.

This provides a robust, simple, and fast training paradigm for *O. vulgaris* (Shomrat *et al.*, 2008) and I chose it as a good indicator of behavioural plasticity in *O. vulgaris*. In addition, the effects of social interaction on behavioural response of a solitary animal like octopus could be considered as a behavioural test of adaptation and plasticity of innate fear.

Finally, my ultimate goal was to set up a series of tools and experimental approaches that may help in increasing the utility of *O. vulgaris* and other cephalopods in the analysis of the

biological machinery underlying learning and memory and more generally in behavioural plasticity. To the best of my knowledge, such an analysis is unprecedented for Cephalopod species.

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CHAPTER 2

BEHAVIOURAL STUDIES: FEAR CONDITIONING AND INNATE FEAR

2.1 Introduction

Fear conditioning is a popular protocol to study learning and memory recall in animals. Its popularity is based on many advantages: *i.* the response is quickly learned after a limited number of trials or just after a single conditioning event; *ii.* the memory trace tends to be stable over time; *iii.* it induces a fear response. The fear-induced response is considered to represent a motivational state evolved to protect individuals from danger. Therefore, avoidance responses observed in the laboratory maybe easily considered analogous to similar circumstances animals may experience in the wild (e.g. Ross, 1971).

Despite the great variety of tasks utilized to explore the neural basis of learning capabilities in *Octopus vulgaris* (review in: Sanders, 1975; Borrelli & Fiorito, 2008), avoidance learning has rarely been utilized with octopuses. In reviewing papers published over the last one hundred years exploring octopus' discriminatory capabilities I counted 89 papers, but only ten that used an avoidance-like protocol to train the animal. This contrasts with the large number of studies that utilized fear conditioning in other invertebrate and vertebrate species (*e.g.* Kida *et al.*, 2002; Shumyatsky *et al.*, 2005; Walters, 2005; Azami *et al.*, 2006; Sakurai, 2008). In the octopus, fear conditioning has been mostly explored using natural stimuli (crab) negatively reinforced by an electric shock (table 2.1).

The idea of exploring fear conditioning in the octopus at the Stazione Zoologica emerged during a visit of Professor Hector Maldonado to dr. G. Fiorito, some years ago. Here I will summarize the results of experiments carried out to study learning and memory recall for fear conditioning in *O. vulgaris* that are unpublished. These are based on *i.* the work carried out by two undergraduate students in Fiorito's laboratory who applied this training protocol to a pharmacological approach (De Simone, 1996; Di Dato, 2000) and *ii.* a series of experiments I carried out at the beginning of my PhD project.

Moreover I designed the first behavioural protocol to study the innate fear in *O. vulgaris*. Brain development, activity and behaviour depend on inherited and environmental influences. The activities performed by animals during their lives allow survival and the reproduction of species. When these activities require the involvement of other members

of the same species they become social activities and can alter the immediate and future behaviour of the animal. Different social behaviours involve the production and the reception of signals that are interpreted in a different manner on the base of social context (Engel *et al.*, 1999; Wilson *et al.*, 2003). These signals can be different from species to species and can drive several behavioural responses that are probably within a framework of conserved neural mechanisms. There are increasing opinions that social interaction can alter the gene expression and consequently animal behaviour (Shors *et al.*, 2001; van Praag *et al.*, 2002; Fahrbach *et al.*, 2003; McRobert *et al.*, 2003; Wommack *et al.*, 2003).

Several studies have been carried out in quite a wide number of social species, but what happen in an "asocial" animal?

To test whether interacting with conspecifics in a solitary animal induce a form of innate fear I studied the influence of social interaction on predatory performances of naïve octopuses during acclimatization. The acclimatization is a period of variable length during which the animal is exposed to a novel environment (tanks of laboratory) and presented with a live prey (*e.g.* Boycott and Young, 1955; Messenger and Sanders, 1972; Gutfreund *et al.*, 1996; Palmer *et al.*, 2006). According to Maldonado during this phase animals adapt to captivity, by a process of positive learning (Maldonado, 1965).

O. vulgaris is typically considered a solitary animal (Altman, 1967). The scarcity of reciprocal interactions between octopuses, such as avoidance or physical contact, have led to categorize octopuses as "asocial" (Hanlon and Messenger 1996; Boyle,1980). In natural conditions, they are solitary and territorial, inhabiting dens that may be distributed in clusters (Guerra A., 1981; Mather and O'Dor 1991) that they defend against conspecifics. In my experimental conditions, two octopuses are in visual contact when in each of their tanks a live crab is presented. This experience could activate an innate fear response because each conspecific can be considered potentially dangerous threatening the territoriality and availability of preys. Thus, this experiment explores the influence that social interaction and innate fear can have on a natural response, such as a predatory response, during acclimatization in laboratory conditions.

Table 2.1 - A tabularized overview of the available studies testing *O. vulgaris* capability to learn to avoid a stimulus (natural or artificial). In the table I report: the negative reinforcement (Punishment), the training protocol (fixed number of trials or to criterion), the number of trials required to train the animal (1-continuous indicates in a single continuous exposure) and inter-trial intervals (ITI, in minutes) and the level of memory recall recorded by authors at various time after training (% Correct at testing; Short-term: STM, Medium-term: MTM, and Long-term memory: LTM). n.a.: information not available.

References (Refs): 1. Goldsmith, 1917; 2. Boycott 1954; 3. Wells, 1959b; 4. Wells, 1959a; 5. Maldonado, 1968; 6. Maldonado, 1969; 7. Young, 1970; 8. Ross, 1971; 9. Sanders and Barlow, 1971; 10. Barlow and Sanders, 1974

Stimulus	Punishment	Train. Crit.	Train. Trials	ITI (min)	%Correct at test STM	%Correct at test MTM	%Correct at test LTM (24h)	Refs
Metallic disk	Sea anemone	Fixed	10 x 4 days	5-10	n.a.	100% (3-4h)	n.a.	1
Hermit crab	Sea anemone	Fixed	1 continuous	n.a.	n.a.	n.a.	n.a.	2
Crab	Sea anemone	Fixed	4	n.a.	n.a.	n.a.	n.a.	2
Artificial object	Shock 6V AC	Crit-1	4-5	3-5-20	n.a.	n.a.	n.a.	3
Artificial object	Shock 6V AC	Crit-6	9	5	n.a.	n.a.	n.a.	4
Shuttle box	Shock 10V AC	Fixed	20	5	n.a.	n.a.	n.a.	5
Shuttle box	Shock 10V AC	Fixed	20	5	n.a.	n.a.	n.a.	6
Crab	Shock 8V AC	Fixed	12 x 5 days	5	n.a.	n.a.	n.a.	7
Hermit crab	Sea anemone	Fixed	1 continuous	n.a.	100% (1h)	100% (1-6h)	100%	8
Crab	Shock 4V AC	Crit-1	16	2	68% (1h)	83% (2h)	88%	9
Crab	Shock 10V AC	Crit-1	6-9	0.05-10	n.a.	n.a.	n.a.	10

2.2 Materials and methods

2.2.1 Fear conditioning

2.2.1.1 Animals and general procedures

A total of 132 naive *O. vulgaris* of both sexes (200 – 600 g body weight) were utilized in these experiments (see also Appendix 1 for details). Before the beginning of the experiments, animals were food deprived for 24h.

2.2.1.2 Stimuli and Reinforcements

As artificial stimuli smooth hard plastic balls (4 cm in diameter) were used. They are identical to those utilized in previous experiments (e.g. Fiorito & Scotto, 1992; Borrelli, 2007). A pair of stainless steel electrodes protrudes 5 mm from the centre of each ball. Each ball is attached to a transparent plastic rod, 80 cm in length. A cylindrical handle at the other end of the rod allowed the experimenter to manually control positioning and movement of the stimuli. In the great majority of cases (60 %; see paragraph 3.1.2) a red ball was utilized as the conditioning stimulus (dark shade; training phase) and a white ball (light shade) for pre-training. Artificial stimuli were reinforced according to experimental conditions. During pre-training, the octopus was rewarded with a small piece of anchovy (average weight: 0.5 g) attached to the electrodes of the balls. During the training phase, the animals were punished by a mild shock (12 V AC, duration: 2 - 3 sec) delivered through the electrodes by pressing the contact by the experimenter as soon as the animal seized the stimulus.

During testing, no reinforcements (either negative or positive) were associated with the artificial stimulus.

The procedure I utilized present stimuli to animals was derived from the original protocols to train octopuses developed by Boycott and Young (1950) with some modifications. Stimuli are landed at approximately 80 cm from the animal's resting position (the den) and in the centre of the tank and are moved in order to elicit the animals' response. Stimuli were presented to the animals inserted with the electrodes facing the frontal glass wall of the tank in order to minimize the view of the reinforcement. A trial started when the experimenter introduced the stimulus and ended when the animal pounced on it.

Once the stimulus landed on the bottom of the tank, it was kept still, in position, for approximately 10 sec. In the absence of a response by the animal, it was moved by the experimenter (one movement per second) first in place and then backwards and forwards with an up-down movement of the object within two centimetres from the bottom of the tank. The movement of the stimulus up and down elicits an approach by the octopus to

approach it. Since the avoidance protocol requires that octopus stop attacking the negative stimulus, a cut-off latency of 60 seconds was set as maximum duration of the trial (during training and testing phases).

2.2.1.3 Experimental procedures

The avoidance protocol includes different phases carried out during three consecutive days (Figure 2.1). For each phase a stimulus was presented to animals in consecutive trials.

Day One - pre-training. The stimulus was applied in order to familiarize the animal with artificial stimuli. During pre-training a positively reinforced ball is presented to animals over a series of trials arranged in one single session. *O. vulgaris* move out of its den in reaction to the presence of the ball, reached the stimulus and then pounced on it. Food (i.e. anchovy) was eaten during the retreat or when the animal was back in its den. The pre-training phase was interrupted once octopuses promptly attacked the ball (*i.e.* within 20 seconds from its appearance in the tank) for six consecutive trails

Day two - training. The animal is presented with another artificial stimulus. This time every attack (or contact) was punished. In this case and at its first contact with the stimulus, the experimenter delivered the shock. The animal reacted to the shock by leaving the object abruptly and going back towards the den[1]. Presentation of the negatively reinforced stimulus continued up to when animals did not touch the ball for at least 6 consecutive trials (training criterion).

Day three: testing phase. Octopuses were tested 24 hours after training for their memory recall. During testing, the octopus was presented with the same stimulus utilized during the training phase (unless otherwise specified), but without reinforcement. In the great majority of cases, animals "froze" at the sight of the stimulus and for the entire trial duration (60 sec), thus showing a good recall of the negative experience. In other cases, the octopus failed to avoid the stimulus; the animal reached (and touched) the ball; in these cases the stimulus was quickly removed by the experimenter (no shock delivered). The amount of memory recall was assessed over a series of 5 consecutive trials.

Figure 2.1: Cartoon depicting phases, timing and procedure of fear conditioning experiment.

1 In some instances, the octopus remained attached to the ball by the action of the suckers; the simultaneous removal of the stimulus from the animal grab by the experimenter facilitates the animal to leave the object

2.2.1.4 Experimental designs

2.2.1.4.1 Experiment 1: testing the robustness of avoidance learning in *O. vulgaris*

A first series of experiments were designed to test whether octopus may learn to avoid an artificial stimulus and if this learning is stimulus specific or may be due to a motivational decline. The experiment was designed according to the original protocol applied by Sanders and Barlow (1971) using crabs (i.e. natural stimulus).

Forty *O. vulgaris* were randomly assigned to six conditions as summarized below:

	Conditions[1]	N	pre-training	Training	test
Experimental	A	8	None	R-	R
	B	12	W+	R-	R
	C	5	R+	W-	W
	D	5	W+	R-	W
Control	E	5	R+	W-	R
	F	5	none	T-	R

R: red ball; W: white ball; T: transparent ball

Further eight animals were utilized as untrained controls. In this case - after pre-training (W+) - animals were presented to the red ball for a series of 15 unreinforced trials.

The experimental conditions differed in that animals were pre-trained or not before the training phase, and that the shade (white/red) of the ball was utilized as stimulus during the various phases of the experiment.

In all conditions stimuli were presented to octopuses with an inter-trial interval (ITI) fixed to 120 sec; this was determined on the basis of the findings of Barlow & Sanders (1974) who reported amelioration of recall with ITI fixed between one and two minutes.

2.2.1.4.2 Experiment 2: testing the effects of ITI on rate of learning and memory recall of an avoidance task

In a second set of experiments I tested the influence of changes in the inter-trial interval (ITI) on the learning and retention performance of octopuses. In this case, the octopuses (N = 92) were pre-trained (W+) and the red ball was utilized as negative stimulus (R-). Again the criterion for training was fixed (no attack over 6 successive trials); in addition, at the end of

the training, animals were presented with a live crab to control for any motivational decline after the avoidance experience.

O. vulgaris were randomly assigned to five experimental groups with ITIs ranging from 60 sec to 20 min (ITI: 1, 2, 5, 10, 20 minutes respectively). Twenty-four hours after training, the octopuses were tested for memory recall; the red stimulus was presented (without reinforcement) for 5 consecutive trials at similar intervals utilized during training.

2.2.2 Innate fear (i.e. testing the effects of social interaction in a solitary animal)

2.2.2.1 Animals and general procedures

A total of 22 naive *O. vulgaris* of both sexes (200 – 400 g body weight) were utilized in these experiments (see also Appendix 1 for details).

2.2.2.2 Experimental procedures and design of experiment 3

Experiments were conducted in five days and consisted mainly of measuring predatory responses in both isolated animals (acclimatization group) and animals which visually interact with a conspecific (social group; figure 2.2). During first day of the experiments on arrival in the laboratory, each animal of both experimental groups was numbered, sexed, weighed, and housed in an experimental tank (for details see Appendix 1). During the second day octopuses of the social group were housed in contiguous tanks and allowed to visually interact with another conspecific removing the opaque partition that impaired the visual interaction of social pairs. The social interaction lasted for four days. Every morning during experimental days (2 – 5 days) the octopuses of both experimental groups were tested for readiness to attack. The octopuses were presented with a live crab attached to a cotton thread that was pulled up before the octopus could seize the prey or anyway after 306 s (ceiling latency). The latency to attack was calculated for each animal as the time elapsed from the first appearance of the crab on the water surface to just before the octopus seizes the prey.

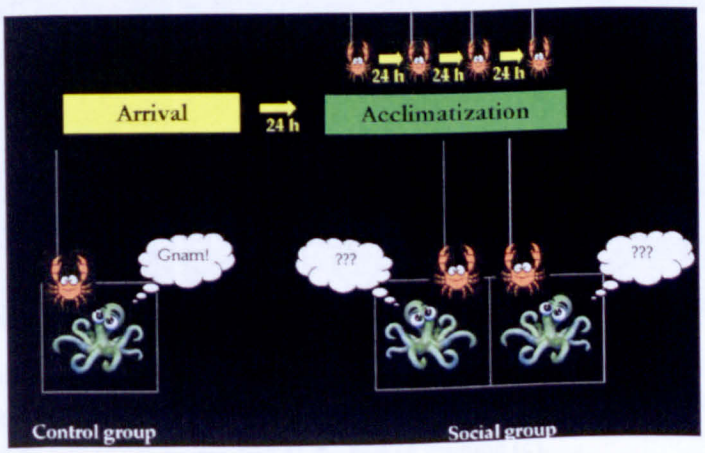


Figure 2.2: Cartoon depicting phases, timing and procedure of innate fear experiment.

2.3 Results

2.3.1 Results of experiment 1 (testing the robustness of avoidance learning in *O. vulgaris*)

Pre-training (B, C, D, E groups) was successful for all octopuses; animals reached the fixed criteria in less than ten trials. As shown in Figure 2.1, during training, the willingness to attack the artificial stimulus decreased due to the negative reinforcement they received. This contrasts with the limited extinction to respond in untrained controls. At the beginning of the training, the octopus readily attacked the ball but, as soon as it perceived the shock, it released the stimulus (see above) withdrawing into the den. As training proceeded, most animals exhibited a variety of cautious or conflict behaviours towards the ball (for detailed behavioural description see Borrelli, 2007). Finally, by the end of the experiment, all the octopuses avoided to respond to the ball (No attack) within the one-minute interval. Animals not responding to the stimulus for the entire trial were scored for a ceiling latency of 61 s (Figure 2.3).

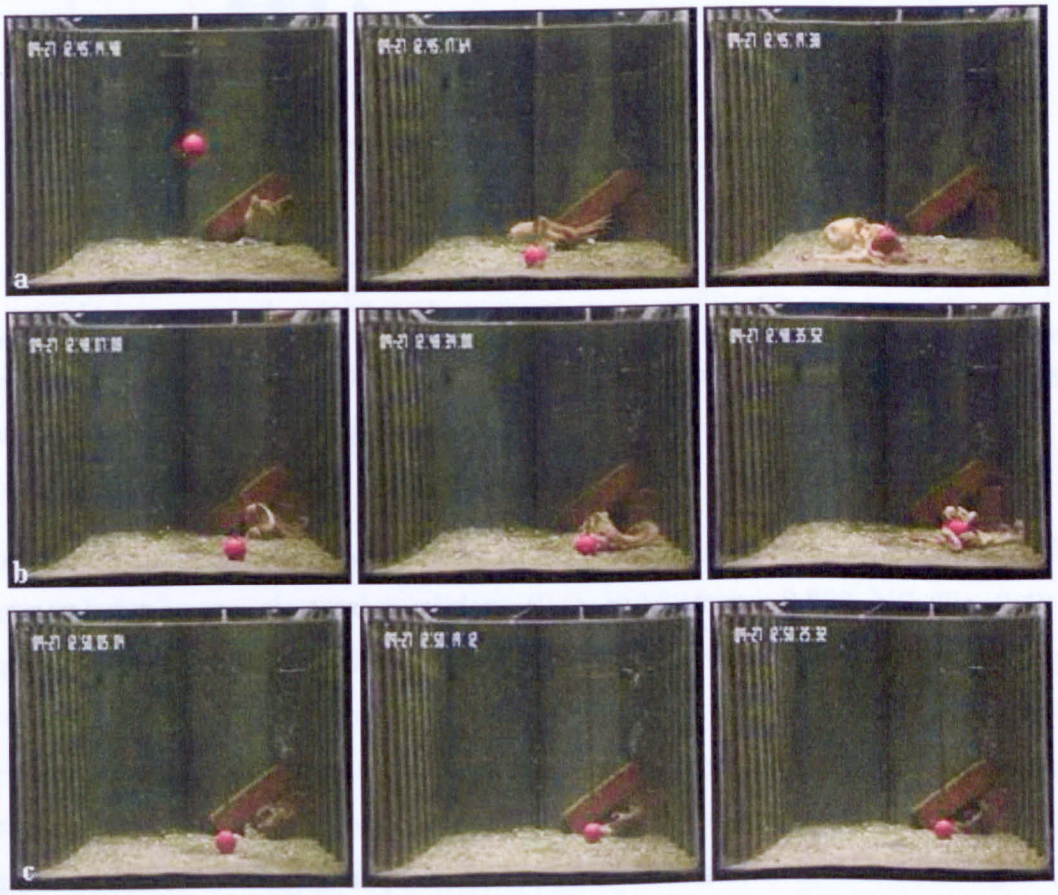


Figure 2.3: Frames taken from video-recordings of the fear conditioning experiment. At the beginning of training octopuses attack the ball and receive the shock (a); as training proceeds animals become more cautious (b), and reach criterion by not attacking the ball at the end of training (c).

Performance in terms of latency to attack (i.e. timing of the response) is not considered in this thesis. A detailed description of the behavioural changes occurring during training have been discussed by Borrelli (2007) and will be part of a behavioural study not considered relevant for the aims of my PhD and therefore not included in this thesis.

Since, a single negative response towards the ball could be due to a motivational decline to attack, all the octopuses were trained to criterion, i.e. training continued until the octopus did not respond to the ball for 6 consecutive trials.

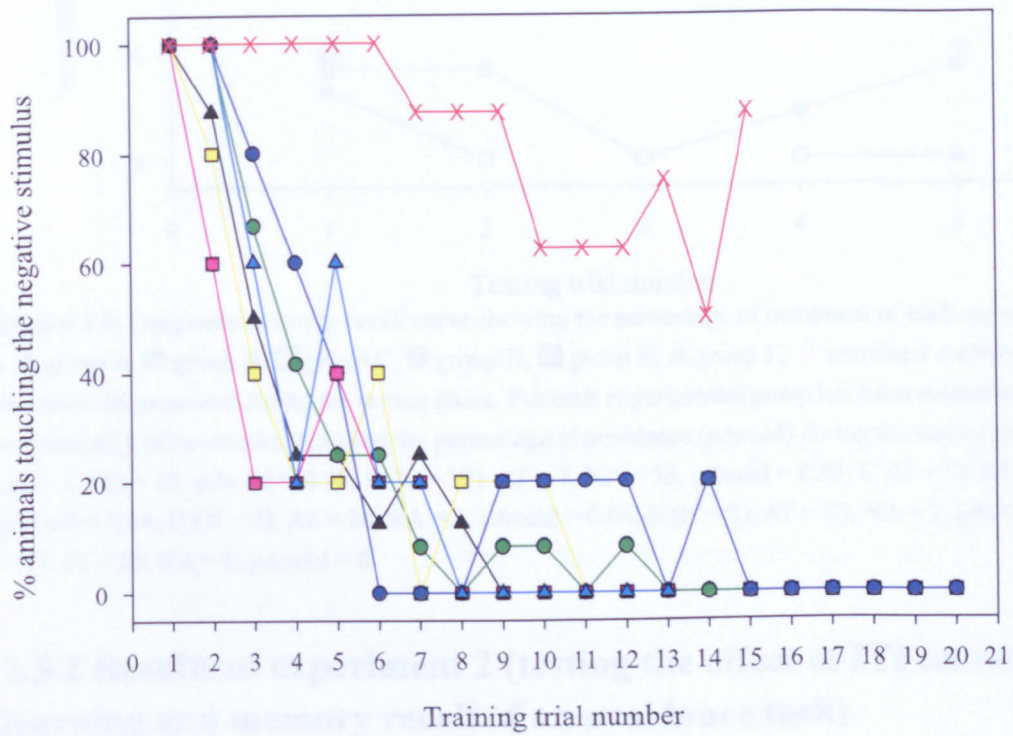


Figure 2.4: Learning curves showing the percentage of octopuses of each experimental group (▲ group A, ● group B, □ group C, ● group D, ■ group E, △ group F, X: untrained controls) that attacked the negative stimulus from first until last trial of training phase.

All animals learned to avoid the artificial stimulus in about 15 trials without a significant difference between experimental conditions ($F_{(5,39)} = 0.336$, $p = 0.888$, NS; Fig. 2.4). The criterion of 6 consecutive trials during which animals did not attack the stimulus, was a good way to measure recall in the short-term. Twenty-four hours later *O. vulgaris* were tested for their memory recall. Similarly to what occurred in the short-term, most of the animals of the experimental conditions (groups A, B, C) did not attack the stimulus. On the other hand, octopuses trained as control (groups D, E, F) attacked the ball almost always (Fig 2.5). As a result, no significant differences emerged when the memory recall shown by octopuses of the experimental or control conditions was compared. Therefore, control and experimental conditions resulted to be significantly different ($F_{(5,39)} = 112.718$, $p < 0.001$).

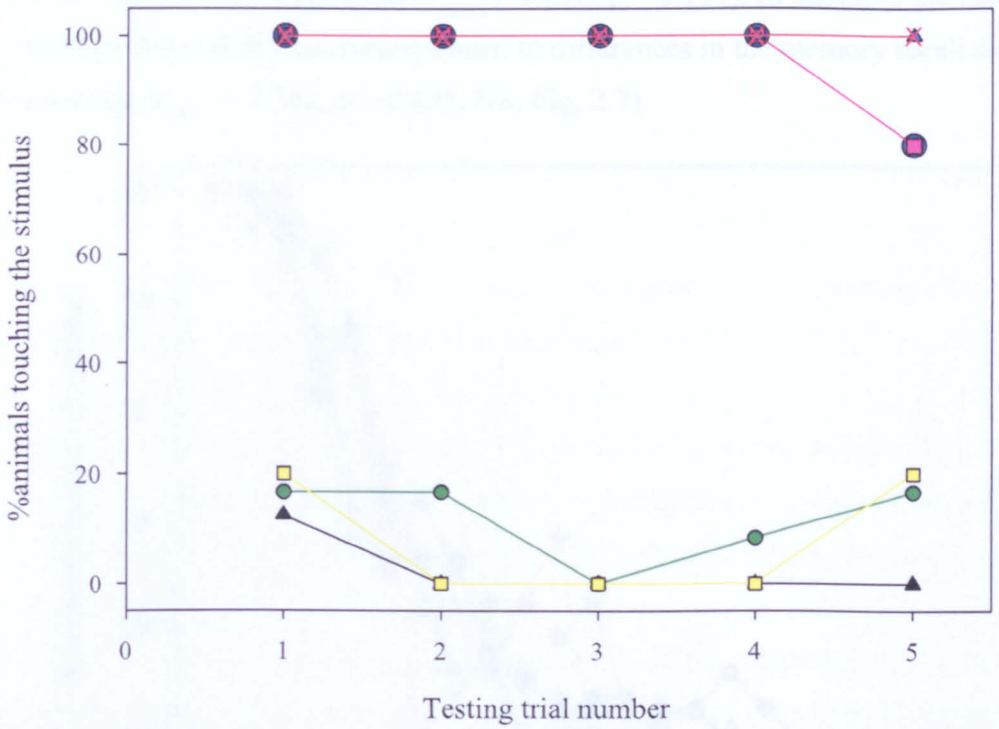


Figure 2.5: Long-term memory recall curve showing the percentage of octopuses of each experimental group (▲ group A, ● group B, □ group C, ● group D, ■ group E, ▲ group F,: X untrained controls) that attacked the stimulus presented during the testing phase. For each experimental group has been measured the number of attacks (AT), of no-attacks (NA) and the percentage of avoidance (pAvoid) during the testing phase. A (N = 8): AT = 1, NA = 39, pAvoid = 0.98; B (N = 12): AT = 7, NA = 53, pAvoid = 0.93; C (N = 5): AT = 2, NA = 23, pAvoid = 0.84; D (N = 5): AT = 24, NA = 1, pAvoid = 0.04; E (N = 5): AT = 23, NA = 2, pAvoid = 0.08; F (N = 5): AT = 25, NA = 0, pAvoid = 0.

2.3.2 Results of experiment 2 (testing the effect of ITI on rate of learning and memory recall of an avoidance task)

Similarly to what occurred in the first set of experiments, after successful pre-training (about 10 trials) octopuses learned to avoid the red ball within a total of 14 trials and achieved a recall of the avoidance experience of about 85% as summarized below.

	Conditions[2]	N	Total number of trials ± SE	Percentage of avoidance
Experimental	ITI 1 min	16	14.75 ± 1.03	83%
	ITI 2 min	18	13.06 ± 0.71	87%
	ITI 5 min	38	12.53 ± 0.62	86%
	ITI 10 min	12	12.73 ± 1.00	90%
	ITI 20 min	8	14.88 ± 0.83	80%

In particular, some differences in performance over training and testing emerged when different ITIs are taken into account. Although the learning curves appear similar (Fig 2.6), the rate of acquisition of the avoidance task is different when shorter and longer ITIs are

compared, although not significant ($F_{(4,91)} = 1.692$; $p = 0.159$). In addition, the changes in the inter-trial interval did not corresponded to differences in the memory recall measured during testing ($F_{(4,91)} = 0.362$, $p = 0.835$, NS; Fig. 2.7).

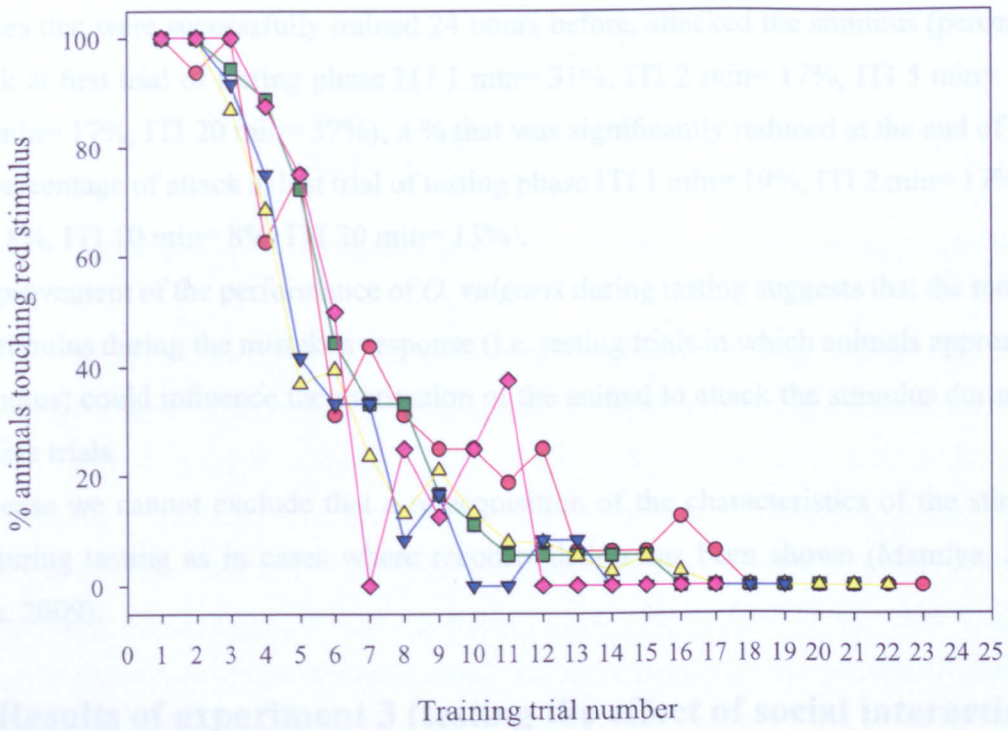


Figure 2.6: Learning curves showing the percentage of octopuses of each experimental group (ITI 1 min, ITI 2 min, ITI 5 min, ITI 10 min, ITI 20 min) that attacked the negative stimulus from first until last trial of training phase.

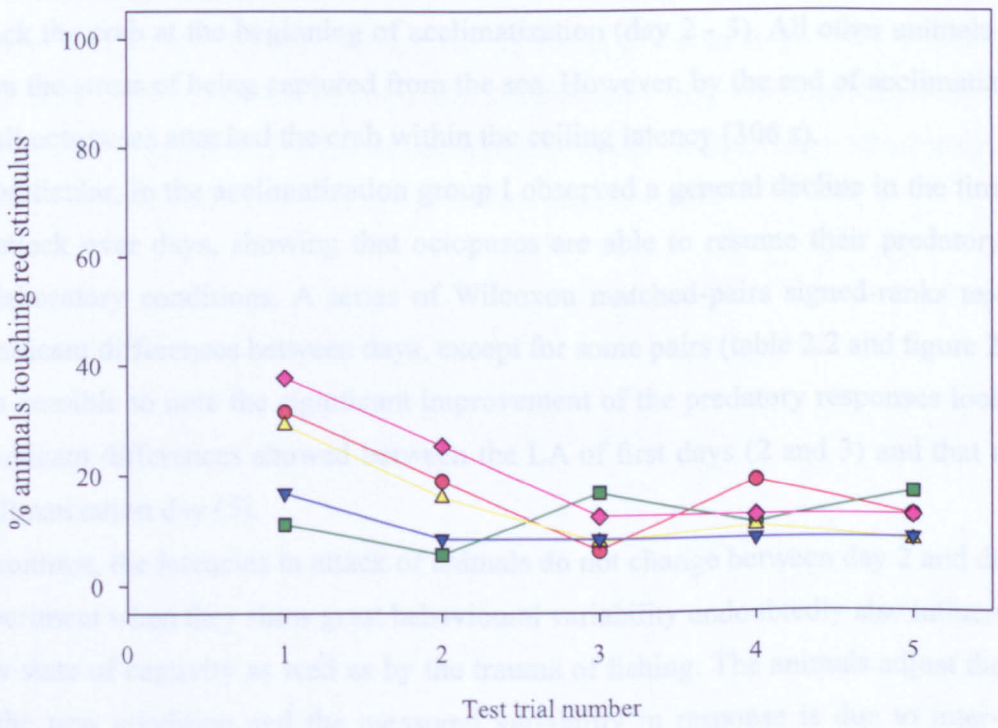


Figure 2.7: Long-term memory recall curve showing the percentage of octopuses of each experimental group (ITI 1 min, ITI 2 min, ITI 5 min, ITI 10 min, ITI 20 min) that attacked the stimulus presented during the testing phase. For each experimental group has been measured the number of attacks (AT), of no-attacks (NA) and the percentage of avoidance (pAvoid) during the testing phase. ITI 1 min (N = 16): AT = 14,

NA = 66 pAvoid = 0.83; ITI 2 min (N = 18): AT = 11, NA = 79, pAvoid = 0.87; ITI 5 min (N = 38): AT = 27, NA = 163, pAvoid = 0.86; ITI 10 min (N = 12): AT = 6, NA = 54, pAvoid = 0.90; ITI 20 min (N = 8): AT = 8, NA = 32, pAvoid = 0.80.

However, it should be noted that during the first trial of the testing phase about 26% of octopuses that were successfully trained 24 hours before, attacked the stimulus (percentage of attack at first trial of testing phase ITI 1 min= 31%, ITI 2 min= 17%, ITI 5 min= 29%, ITI 10 min= 17%, ITI 20 min= 37%); a % that was significantly reduced at the end of the 5 trials (percentage of attack at last trial of testing phase ITI 1 min= 19%, ITI 2 min= 17%, ITI 5 min= 8%, ITI 10 min= 8%, ITI 20 min= 13%).

The improvement of the performance of *O. vulgaris* during testing suggests that the removal of the stimulus during the mistaken response (i.e. testing trials in which animals approached the stimulus) could influence the motivation of the animal to attack the stimulus during the successive trials.

In this case we cannot exclude that a re-acquisition of the characteristics of the stimulus occur during testing as in cases where reconsolidation has been shown (Mamiya, 2009; Monfils, 2009).

2.3.3 Results of experiment 3 (testing the effect of social interaction in a solitary animal)

Only two octopuses (one from the acclimatization and one from the social group) did not attack the crab at the beginning of acclimatization (day 2 - 3). All other animals recovered from the stress of being captured from the sea. However, by the end of acclimatization (day 5) all octopuses attacked the crab within the ceiling latency (306 s).

In particular, in the acclimatization group I observed a general decline in the time required to attack over days, showing that octopuses are able to resume their predatory response in laboratory conditions. A series of Wilcoxon matched-pairs signed-ranks tests showed significant differences between days, except for some pairs (table 2.2 and figure 2.5).

It is possible to note the significant improvement of the predatory responses looking at the significant differences showed between the LA of first days (2 and 3) and that one of last acclimatization day (5).

In contrast, the latencies to attack of animals do not change between day 2 and day 3 of the experiment when they show great behavioural variability undoubtedly also influenced by the new state of captivity as well as by the trauma of fishing. The animals adjust their activity to the new condition and the measured variability in response is due to inter-individual differences.

Table 2.2: Results of Wilcoxon matched-pair signed-ranks tests for the acclimatization group.

	Z	P
day 2 - day 3	1.020	0.308
day 2 - day 4	1.138	0.255
day 2 - day 5	2.001	**0.045**
day 3 - day 4	3.061	**0.002**
day 3 - day 5	2.981	**0.003**
day 4 - day 5	1.201	0.230

Octopuses of social group did not show a similar amelioration of their latency to attack over days.

In fact, the predatory latencies of animals do not appear to change significantly in the different experimental days except on day 2 and day 4 (Wilcoxon signed-ranks tests: $Z = 2.314$, $P = 0.021$; figure 2.8). Although even in this group as well as in the control group may be possible to observe in the last two days (day 4 and day 5) a decrease of behavioural variability of animals that denotes an uniform response to adapt to experimental conditions. The comparison between the predatory performances during acclimatization of the animals belonging to experimental (social) and control (acclimatization) groups provided significant differences in the day 4 (Mann-Whitney test, $Z = 2.647$, $N1 = 10$, $N2 = 12$; $P = 0.007$; figure 2.8) and day 5 (Mann-Whitney test, $Z = 2.647$, $N1 = 10$, $N2 = 12$; $P = 0.007$; figure 2.8). These differences could be related to different levels of competition which are between two experimental groups. In fact, the octopuses of control group were in a "safe" environment without competitors instead the animals of social group undergo the conditioning of the sharing the small niche with a conspecific. It is possible to hypothesized that social interaction, in this context, could induce innate fear that may inhibit or interfere with the natural positive learning process shown by animals of control group.

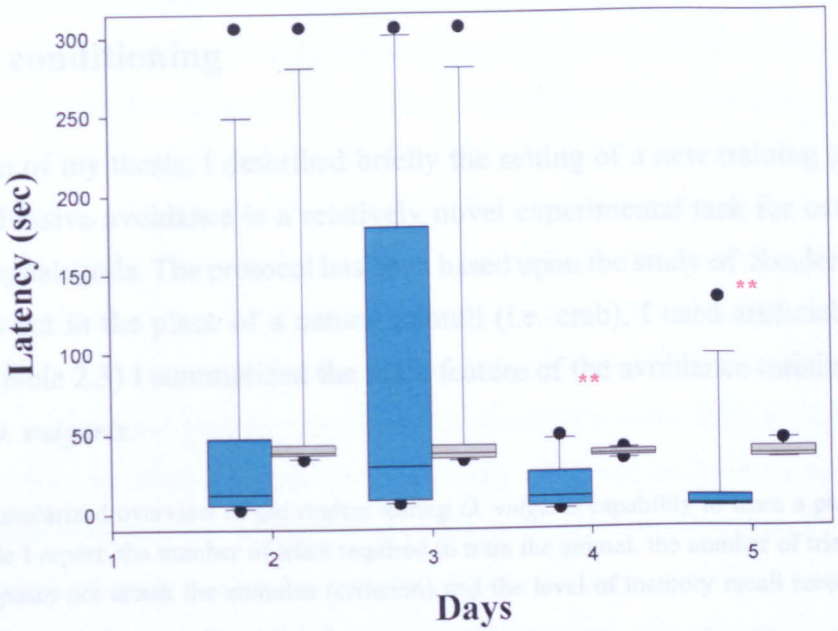


Figure 2.8: In this figure the time to attack (sec) is given for each experimental group in four experimental days. The distribution is shown by green vertical boxes for acclimatization group and gray boxes for social group, respectively. The distribution is shown by vertical box plot as median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by Mann-Whitney test are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

2.4 Discussion

2.4.1 Fear conditioning

In this section of my thesis, I described briefly the setting of a new training procedure for *O. vulgaris*. Passive avoidance is a relatively novel experimental task for octopus and, in general for cephalopods. The protocol has been based upon the study of Sanders and Barlow (1971; 1974) but in the place of a natural stimuli (i.e. crab), I used artificial ones. In the table below (table 2.3) I summarized the main feature of the avoidance training procedures utilized for *O. vulgaris*.

Table 2.3 - A tabularized overview of the studies testing *O. vulgaris* capability to learn a passive avoidance task. In the table I report: the number of trials required to train the animal, the number of trials of training in which the octopuses not attack the stimulus (criterion) and the level of memory recall recorded by authors during testing phase (percentage of avoidance).

Experimental group	Total number of trials		Trials to criterion		Percentage of avoidance (24h)	
	Crab	Artificial stimulus	Crab	Artificial stimulus	Crab	Artificial stimulus
Sanders and Barlow 1971	16		1		86%	
Barlow and Sarders 1974 ITI: 0.05 min	9		1		n.a	
Barlow and Sarders 1974 ITI: 0.5 min	9		1		n.a	
Barlow and Sarders 1974 ITI: 1 min	6		1		n.a	
Barlow and Sarders 1974 ITI: 2 min	6		1		n.a	
Barlow and Sarders 1974 ITI: 5 min	7		1		n.a	
Barlow and Sarders 1974 ITI: 10 min	8		1		n.a	
A		11		6		98%
B		12		6		93%
ITI: 1 min		15		6		83%
ITI: 2 min		13		6		87%
ITI: 5 min		13		6		86%
ITI: 10 min		12		6		90%
ITI: 20 min		15		6		80%

During this experiment octopuses were induced to differentially respond to an artificial stimulus. After a short period of acclimatization to laboratory conditions octopuses showed stimulus generalization by attacking an artificial stimulus (never seen before) with a performance comparable to that exhibited towards a natural prey. In fact, during the pre-training phase and with the progression of trials, the performances of the animals improved. They showed a progressively shorter latency to attack plastic ball until to reach the criterion

(six consecutive attacks with the latency to attack shorter than 20 sec). This quick response was also facilitated by the presence of reward (i.e. a piece of anchovy) attached to the stimulus (as occurs for Borrelli, 2007). During the training phase, the animals were presented with another artificial stimulus different from that one used in the pre-training phase (contrary to what occurred in Borrelli, 2007). Any contact of octopuses with the stimulus was punished with an electric shock. Thus, during the first trial the animals promptly attacked the stimulus (the punishment was unexpected), but with the progression of trials and after some negative experiences we observed a reduction in the animals' propensity to attack the artificial stimulus. The initial readiness to attack was substituted by fear. Animals appeared more cautious towards the stimulus until they consistently avoided it (reaching the criterion of six consecutive no attacks). In the great majority of cases, the training experience with repeated punishments did not affect the animal's motivation to attack; in fact, the trained octopuses responded quite readily to a live crab presented at the end of the training (data not shown), resuming their predatory behaviour. The employment of a natural prey as a stimulus to avoid (Sanders and Barlow, 1971) makes the memory retention more weak and erasable. In contrast, in our conditions a robust and stable memory trace was recorded. In addition, avoidance is a stimulus specific response.

Learning to avoid a negatively reinforced stimulus (i.e. a plastic object) occurred, in these experiments, independently from characteristics of the object (red, white or transparent) and the fact that animals were pre-trained or not. A single session of training of about 20 trials was enough for octopuses to reach the criterion and learning that an artificial stimulus may deliver a potential danger. The level of learning induced a robust short-term memory recall: no attacks were observed during the last 6 trials of training (i.e. criterion trials). It is interesting to note that during the "criterion trials" *O. vulgaris* stayed motionless at home in some cases withdrawing when the ball was placed in their surroundings (the ball was kept at least 10 cm away from octopus' home position) and paling or with a light mottle appearance. In some other instances flushing towards the stimulus with a jet of water from the siphon was observed. My data parallel the results obtained by previous studies in the sense that octopuses can be trained in a single session of massed trials (about 20 trials, spaced at short intervals; for tactile learning: Wells and Young, 1970; for visual learning: Sanders and Barlow, 1971).

In summary, octopuses: *i.* learned in a limited amount of trials to avoid an artificial stimulus; *ii.* their learned performance was stable in the short-term (criterion trials) and 24 hours afterwards (testing phase); *iii.* their correctness was above 80%, and *iv.* learning appeared to be stimulus specific: octopuses trained to avoid to a given stimulus (e.g. red ball) and responded to the other object (i.e. white ball) during testing.

Again these results parallel those of Sanders and Barlow (1971) in training *O. vulgaris* to

avoid a natural stimulus (i.e. a crab tied to a transparent rod). However, it is noteworthy to underline some procedural differences between the two sets of experiments. These differences can be summarized as below:

1. we utilized animals that were well customized to laboratory conditions (data are not available for Sanders and Barlow, 1971) to stabilize predatory performance and stimulus generalization among different individuals;
2. we used an artificial stimulus conditioned to avoid interference with species-specific predatory behaviour and oscillations in performance due to motivation;
3. we utilized a higher voltage as shock (12 V AC vs 4V AC); I cannot exclude that the differences, in my conditions, may cause a faster rate of training and a better recall;
4. the criterion utilized by the two sets of experiments is different: in these experiments, six consecutive no-attack trials are used, while previous authors used a single trial as criterion for measuring learning;
5. memory recall was tested without any reinforcement (in our case) or as number of trials of savings during retraining at different intervals (from 30 to 1800 min, Sanders and Barlow, 1971).

Despite such differences, avoidance of the stimulus was achieved in a similar number of trials (this study vs. Sanders and Barlow, 1971: 15 vs. 16 trials on average, respectively) and with a similar performance measured at recall (about 80% of avoidance).

During the first series of experiments reported here, the training trials were separated by two minutes of inter-trial interval as suggested by Barlow and Sanders (1974). The authors demonstrated that octopuses trained on a passive avoidance task by using a "paled" crab as *discriminandum* reached the criterion in fewer trials when trained with a 1-2 min ITI than other octopuses (with ITI ranging from 30 to 180 sec); moreover, they suggested that longer intervals (ITI 5, 10 min) do not improve learning rate. During the second series of experiments, I tested the influence that the variation of ITI duration could have on the learning rate and on memory recall. As suggested by Barlow and Sanders (1974) the spacing of training trials with longer ITI neither improve the learning performances nor affect the retention.

These results seem to contrast what has been demonstrated in the honeybee (Toda *et al.*, 2009). In these circumstances, honeybees improved both acquisition and retention when ITIs ranged from 1 to 3 or 5 minutes. Such differences in the behavioural responses of different species over similar paradigms could be related to the differences among the behavioural paradigms as well as the relevance of stimulus adopted.

In addition, I cannot exclude that the differences in the memory retention induced by variation of ITI could not be appreciable at 24 hours after training but could be measurable only after a longer time period as demonstrated by Menzel and co-workers (2001) for the honeybee.

2.4.1.1 Departures from original protocol

This protocol for testing fear conditioning in *O. vulgaris* has been utilized with some variants to explore the role of neural circuitry for learning (Shomrat *et al.*, 2008) and for exploring the expression of some target genes in different regions of the brain of the animals after learning (this PhD).

In particular, Shomrat and co-workers (2008) trained octopuses using an ITI fixed to 5 min and with a shorter training criterion (4 consecutive trials, crit-4). This had the advantage of reducing the total duration of the training sessions by about 12 minutes. This change did not seem to affect the learning rate. All animals of both experimental groups (crit-4 and crit-6) reached the criterion in 14 trials. However, these changes resulted in an important difference in the level of memory recall. In fact, 80% of animals trained with crit–4 attacked the negative stimulus during the first trial of the testing phase (Shomrat *et al.*, 2008). This contrasts with less than 30% of octopuses that failed to recall the task at the first testing trial after being trained with crit-6 (figure 2.2). Significant differences could be detected also during the other test trials. The comparison between criterion-4 and criterion-6 test results: at second trial 60% *vs* 16% of animals that attacked; at third trial 15% *vs* 8%; at fourth trial 40% *vs* 11%; fifth trial 60% *vs* 8%.

In this thesis I decided to use a slightly modified protocol. The pre-training phase was shortened to 3 fixed trials. Only the animals that responded with prompt attacks (less than 10 sec) to three successive stimulus presentations were trained after thirty minutes. The training phase consisted of successive negative stimulus presentations spaced by 1 minute of ITI (see above that no difference emerged between longer and shorter ITI intervals). The training phase was interrupted when the animals reached a criterion of four consecutive no attacks (crit–4; figure 2.9). In addition, twenty-four hours after training the octopuses were tested by a single trial.

Similarly to what occurred during experiments carried out by Shomrat and co-workers (2008) the variation of the number of criterion trials affected retention (figure 2.10). In fact, the 67% of octopuses trained at criterion-4 attacked the stimulus compared to the 30% of octopuses trained at criterion-6.

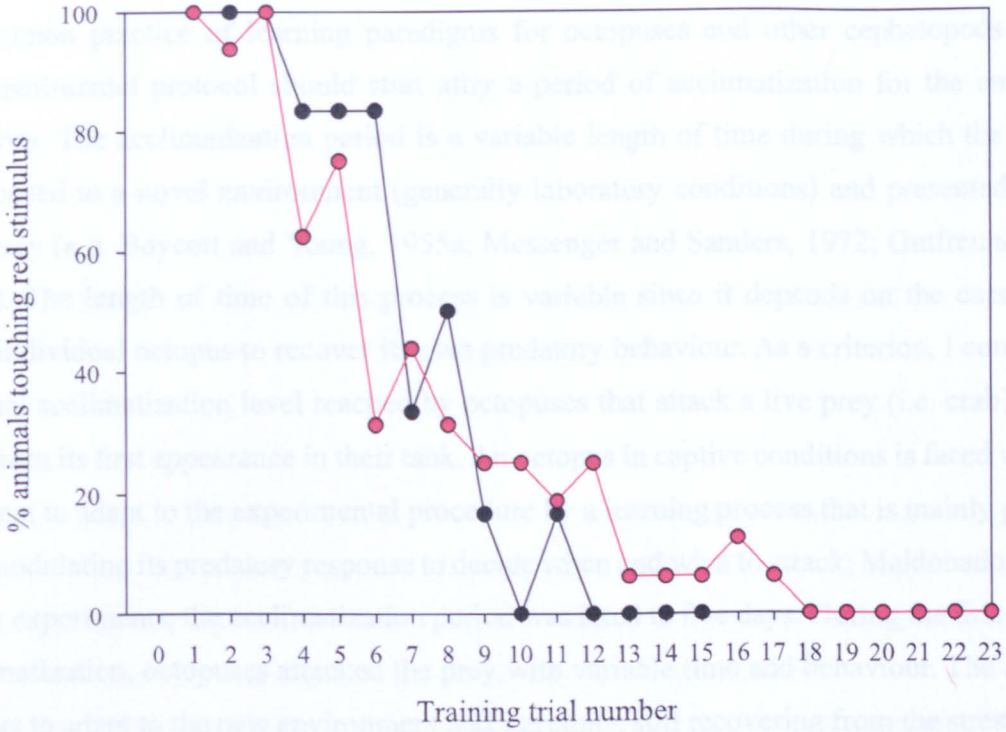


Figure 2.9: Learning curves showing the percentage of octopuses of two experimental groups with octopuses trained with ITI = 1 min (red circle: crit-6 ; black circle: crit-4) that attacked the negative stimulus from first until last trial of training phase.

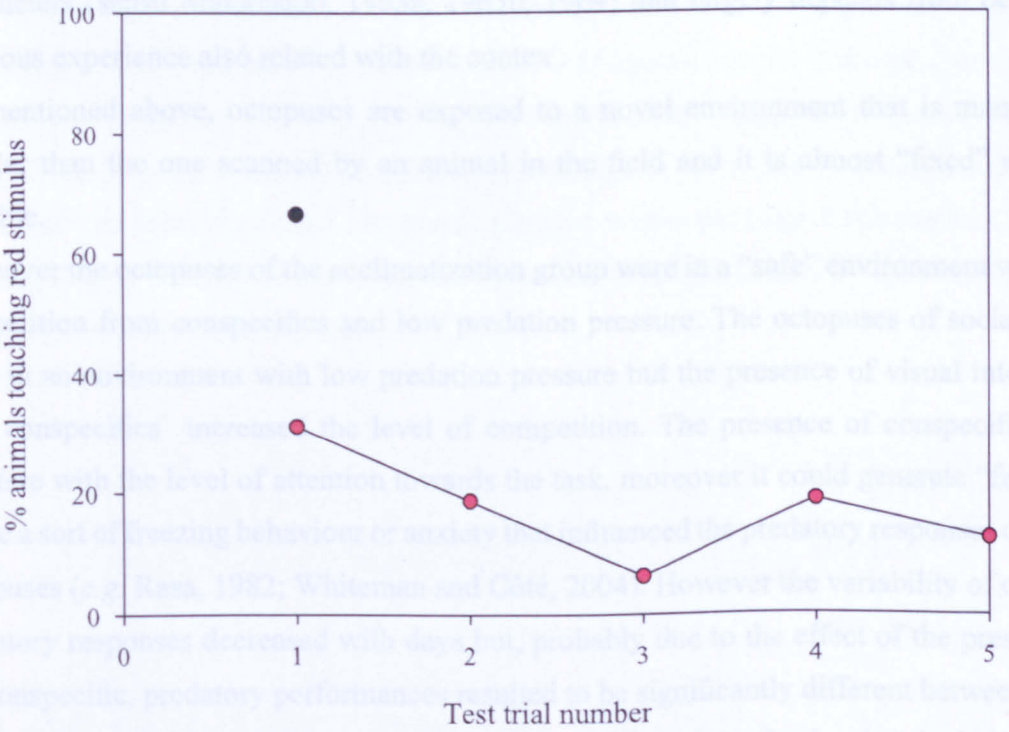


Figure 2.10: Long-term memory recall curve showing the percentage of octopuses of two experimental groups with octopuses trained with ITI = 1 min (red circle: crit-6 ; black circle: crit-4) that attacked the stimulus presented during the testing phase. For each experimental group has been measured the number of attacks (AT), of no-attacks (NA) and the percentage of avoidance (pAvoid) during first trial of testing phase. ITI 1 min crit-6 (N = 16): AT = 5, NA = 11 pAvoid = 0.69; ITI 1 min crit-4 (N = 6): AT = 4, NA = 2, pAvoid = 0.33

2.4.2 Innate fear

A common practice of learning paradigms for octopuses and other cephalopods is that an experimental protocol should start after a period of acclimatization for the animal in captivity. The acclimatization period is a variable length of time during which the animal is exposed to a novel environment (generally laboratory conditions) and presented with a live prey (*e.g.* Boycott and Young, 1955a; Messenger and Sanders, 1972; Gutfreund *et al.*, 1996). The length of time of this process is variable since it depends on the capacity of each individual octopus to recover its own predatory behaviour. As a criterion, I considered optimal acclimatization level reached by octopuses that attack a live prey (i.e. crab) within 20 s from its first appearance in their tank. An octopus in captive conditions is faced with the problem to adapt to the experimental procedure by a learning process that is mainly positive (*i.e.* modulating its predatory response to decide when and what to attack; Maldonado, 1963). In my experiments, the acclimatization period was fixed to five days. During the first days of acclimatization, octopuses attacked the prey with variable time and behaviour. The animals had yet to adapt to the new environment and, certainly, still recovering from the stress due to capture from the sea and subsequent transportation to the tanks of the laboratory. However, the reverse occurred by the end of acclimatization. Once octopuses well adapted to the captivity animals' predatory response resulted to be controlled by the so-called empirical parameters (sensu Maldonado, 1963a, 1963b, 1964) that largely depends from octopuses previous experience also related with the contex .

As mentioned above, octopuses are exposed to a novel environment that is many times smaller than the one scanned by an animal in the field and it is almost "fixed" and less variable.

Moreover the octopuses of the acclimatization group were in a "safe" environment with low competition from conspecifics and low predation pressure. The octopuses of social group were in an environment with low predation pressure but the presence of visual interaction with conspecifics increased the level of competition. The presence of conspecifics may interfere with the level of attention towards the task, moreover it could generate "fear" and create a sort of freezing behaviour or anxiety that influenced the predatory responses of tested octopuses (*e.g.* Rasa, 1982; Whiteman and Côté, 2004). However the variability of octopus' predatory responses decreased with days but, probably due to the effect of the presence of the conspecific, predatory performances resulted to be significantly different between social and individual groups. For this reason could be possible to hypothesize that the behavioural response of octopuses of social group is influenced by innate fear, a form of fear not acquired by the experience, but naturally present in the octopus, a cryptic and solitary predator.

CHAPTER 3

STATE OF ART OF KNOWLEDGE ON GENES IN CEPHALOPODS

As a second task of my PhD project, I searched for the available knowledge on genes in cephalopod species as a good basis to start molecular studies. This with the aim of *i.* depicting a state of art on this topic and *ii.* facilitating the search for target genes potentially involved in the mechanisms activated during fear responses in *Octopus vulgaris*.

Despite the fact that biologists recognize in cephalopods elevated flexibility and complexity in their motor, sensory, behavioural and cognitive capabilities, only a limited effort has been dedicated in the analysis of the underlying molecular machinery (for review see: Borrelli and Fiorito, 2008). This is a notable exception if compared to other invertebrate taxa.

I searched for the cephalopod nucleotide sequences available in GenBank and found 5926 sequences. Then, I selected few among about 700 known species belonging to the class Cephalopoda (Sweeney and Roper, 1998) and I searched for nucleotide sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). In particular, I choose seven genera on the basis of criteria such as: taxonomic position within the Class Cephalopoda, the fact of being largely implied as model species because of the peculiarity of their biological or physiological characteristics (table 3.1).

Table 3.1 : aim of studies conducted on seven cephalopod genera selected for our research .

Aim of studies	Octopus	Sepia	Loligo	Eledone	Euprymna	Sepiola	Sepioteuthis
Morphological and morphometrical observation	x	x	x	x	x	x	x
Life history	x	x	x	x	x	x	x
Learning and memory	x	x		x			
Organization of CNS, function and development	x	x	x	x	x		x
EST library of EYE	x						
Behaviour	x	x	x		x	x	x
Eye and visual system	x	x	x	x	x	x	x
Arm moviment and motor centers	x						
Body pattern	x	x	x	x	x	x	x
Photoreceptors	x	x	x	x	x	x	
Body structure,	x	x	x	x	x	x	x
Genetic diversity and population structure	x	x	x	x	x	x	x
Gland ink and ink	x	x	x				x
Distribution of some molecules, hormones, genes in the body and in the CNS	x	x	x	x	x	x	x
Cartilage	x	x	x		x		
Statolith	x	x	x	x			x
Swimming dynamics	x	x	x				x
Giant axon and synapse			x				x
Biofilm formation and microbial symbiosis					x	x	
Bioluminescent organs					x	x	
Annotated cDNA library					x		
Tagging	x	x	x				x

This search produced a total of about 1800 nucleotide sequences corresponding to only about 3% of the total number of sequences known for taxon *Aplysia* or honeybee (table 3.2).

Table 3.2: Total number of nucleotide sequences available in GenBank (NCBI) for seven genera of cephalopods (1800 sequences) and some other model invertebrate organisms .

Genus	Total number of nucleotide sequences in NCBI
Cephalopods	
Sepia	529
Sepiola	50
Euprymna	217
Loligo	413
Sepioteuthis	107
Octopus	473
Eledone	25
Other invertebrates	
Aplysia	69482
Lymnea	527
Eisenia	590
Apis	60120

In order to provide a more complete picture on the available knowledge for each genus of interest, the sequences available were first analyzed in order to identify redundancy. I proceeded aligning the core nucleotide sequences coding for the same molecules; sequences that resulted to be part (even with different degree of overlapping) of another sequence found in the database with a different accession number (and coding for the same molecule) were considered redundant.

The tables 3.3 – 3.9 provide the total number of core nucleotide sequences available in GenBank together with the number of redundant and not redundant sequences for each species analyzed.

Subsequently, I analyzed the not-redundant sequences (1542) using the Blast2GO software in order to identify their molecular functions.

Blast2GO (http://www.blast2go.org) consists of five steps: BLASTing, mapping, annotation, statistical analysis and visualization. The BLASTing allows to find sequences similar to query set following Basic Local Alignment Search Tool (Blast hits; http://www.ncbi.nlm.nih.gov/BLAST).

Mapping retrieves GO terms associated to the Blast hits. This process consists of four stages:

i. BLAST results are used to retrieve gene names or symbols of hits; each gene name is searched in the gene-product table of GO database.

ii. BLAST results are used also to retrieve UniProt IDs employing the mapping files from the Non-redundant Reference protein Databases (e.g.: PSD, UniProt, Swiss-Prot, TrEMBL, RefSeq, GenPept and PDB).

iii. accession numbers are used as a query to search directly in the *dbxref table* of the GO database.

iv. accession numbers identified by the blasting process are searched directly in the gene-product of the GO database.

During the annotation step, the software applies different filters to find the most specific GO terms with a high level of reliability for each query sequences.

The two final steps characteristic of this software allowed a statistical analysis and representation of the results of data analysis.

Figure 3.1 summarizes the results of Blast2GO analysis. I analyzed a total of 1534 sequences which provided a total of 1090 annotated sequences.

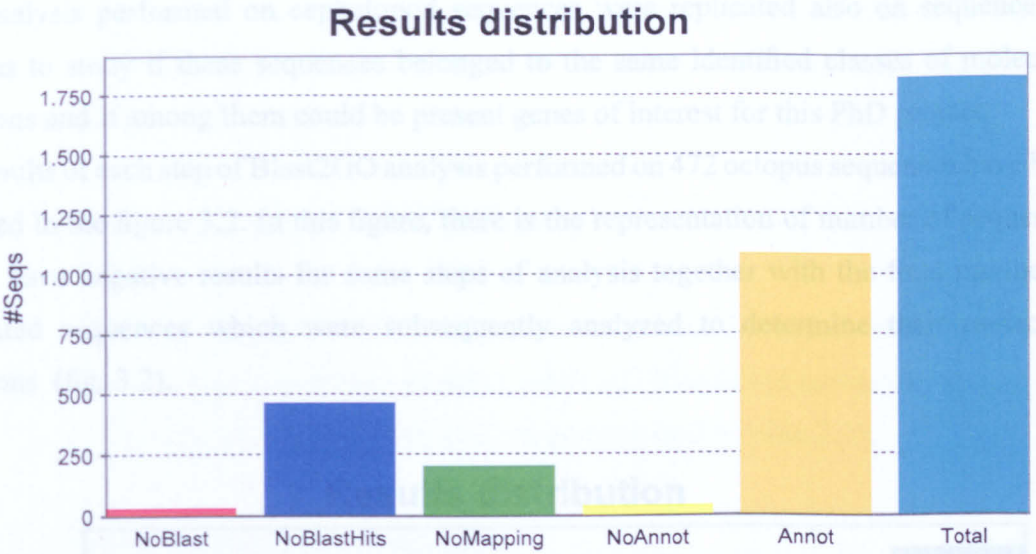


Figure 3.1: Blast2GO analysis distribution of the nucleotide sequences of the seven genera of cephalopods here considered (i.e. *Sepia, Sepiola, Euprymna, Loligo, Sepioteuthis Octopus and Eledone*). First four bars represent the number of sequences which did not provide any result after the analysis. Third and fourth bars are relative to sequences that had not positive results for mapping and annotation steps. Whereas, the last two bars represent the final number of annotated sequences and the total number of sequences analyzed.

I cannot exclude that a quota of not annotated sequences resulted from Blast2GO analysis may be considered as not coding RNA (ncRNA). this should be of potential interest for studies related with modulation and evolution of neural process as appears in the last years (Nakaya *et al.*, 2007, Chodroff *et al.*, 2010). Annotated sequences have been further grouped into twelve classes of molecular functions as shown in table 3.10.

Table 3.10: This table reports the distribution of cephalopod (e.g.: *octopus, sepia, loligo, eledone, euprymna, sepiola and sepioteuthis*) and octopus sequences deposited in NCBI GenBank on the base of their molecular functions.

	Percentage of sequences for each molecular function class	
Molecular functions	**Cephalopoda***	**Octopus**
Binding	34,31%	32,70%
Catalytic activity	31,61%	33,39%
Transporter activity	25,41%	21,92%
Auxiliary transport protein activity	0,12%	
Molecular transducer activity	2,70%	3,94%
Metallochaperone activity	0,05%	
Transcription regulator activity	2,60%	3,25%
Structural molecule activity	2%	4,11%
Motor activity	0,43%	
Antioxidant activity	0,24%	
Enzyme regulator activity	0,28%	
Translation regulator activity	0,24%	0,68%

The analysis performed on cephalopod sequences were replicated also on sequences of octopus to study if these sequences belonged to the same identified classes of molecular functions and if among them could be present genes of interest for this PhD project.
The results of each step of Blast2GO analysis performed on 472 octopus sequences have been reported in the figure 3.2. In this figure, there is the representation of number of sequences which have negative results for some steps of analysis together with the final number of annotated sequences which were subsequently analyzed to determine their molecular functions (fig. 3.2).

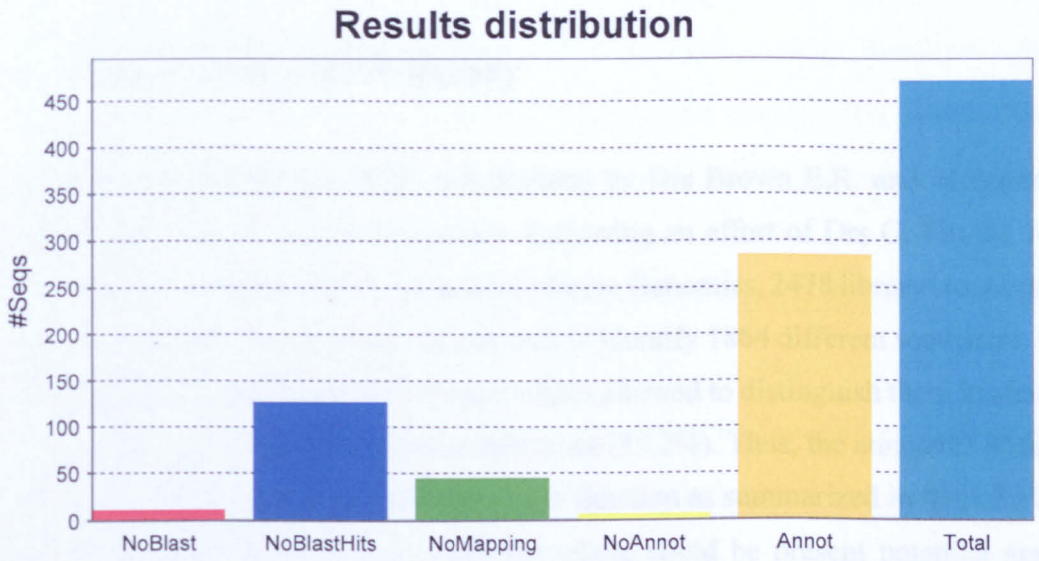


Figure 3.2: This graph reports the results distribution of Blast2GO analysis on octopus sequences deposited in NCBI GenBank. First four bars represent the number of sequences which have no results respectively after Blasting, mapping and annotation processes. Whereas, the last two bars represent the final number of annotated sequences and the total number of sequences analyzed.

The octopus molecules codified by gene sequences of NCBI GenBank resulted involved principally in catalytic activities (33.4% of sequences), in binding processes (32.7% of sequences) and in transport activities (25.4% of sequences) such as the cephalopod sequences above reported. Whereas a minor number of sequences were involved in structural activities of cells (4.1% of sequences), transductional activity (3.9% of sequences) and regulation of transcription (3.2% of sequences), the same categories of molecular functions where the cephalopod sequences above cited were involved. Finally, only the 0.7% of sequences performed the regulation of translation.

3.1 State of art of knowledge on genes in *Octopus vulgaris*

Although unpublished or not deposited in GenBank, I had access to other two sources of

Octopus vulgaris gene sequences: professor T. Gojobori group's EST library enriched in octopus eye structural genes (Ogura *et al.*, 2004) and a cDNA library from octopus brain (Brown and Fiorito, unpublished).

3.1.1 Professor Gojobori's EST library

This library has been sequenced; 2824 sequences have been identified, but only a quarter (24.5%) resulted to belong to known proteins, while 62.7% resulted not-annotated (Ogura *et al.*, 2004). The results of Blast2GO analysis are summarized in table 3.11.

3.1.2 Octopus brain cDNA library

A cDNA library from octopus brain was realized by Drs Brown E.R. and Mohammed I. within the EDD node of Marine Genomics. Following an effort of Drs G. Fiorito, M. Ina Arnone and D. Arendt within the EDD node of Marine Genomics, 2478 library clones (among 80000 clones) have been sequenced and allowed to identify 1864 different sequences. These have been analyzed with Blast2GO software which allowed to distinguish them in annotated (39.6%), obsolete (25.2%) and unknown molecules (35.2%). Thus, the annotated sequences have been analyzed to determine their molecular function as summarized in table 3.11.
Though the presence of functional categories where could be present potential genes of interest (e.g. transport activity, regulation of transcription), only few octopus known genes have been considered suitable for the aim of this project: *α-tubulin*, *octopressin*, *cephalotocin* (from NCBI GenBank) and *stathmin* (from Gojobori's EST library).

Table 3.11: This table reports the distribution of octopus available sequences on the base of their molecular functions.

	Percentage of sequences for each molecular function class		
Molecular functions	***O. vulgaris* GenBank**	***O. vulgaris* EST library**	***O. vulgaris* cDNA library**
Binding	32,70%	25,20%	34,67%
Catalytic activity	33,39%	29,50%	31,25%
Transporter activity	21,92%	5,60%	9,21%
Molecular transducer activity	3,94%	4,95%	4,60%
Transcription regulator activity	3,25%		7,45%
Structural molecule activity	4,11%	34,70%	12,86%
Translation regulator activity	0,68%		

3.2 Conclusions

Contrasting to the large body of dedicated studies in neurobiology, neurophysiology and behaviour, the molecular analysis of genomic information is rather limited for *O. vulgaris*. The only available nucleotide sequences are those present in the GenBank (134 nucleotide sequences). The lack of suitable transcriptome and genome information of *O. vulgaris* is currently a large drawback for the use of this species in functional and comparative molecular studies. For this reason, the access to two new sources (Ogura *et al.*, 2004; Brown and Fiorito, unpublished) of transcript sequences represents first steps towards functional genomic research of *O. vulgaris*. This will allow the use of this animal not only as a model system for various areas of behaviour and neuroscience research but also for general evolutionary and comparative genomics.

The study of these two sources of gene sequences provided the identification of 1577 (648 from EST library and 929 from cDNA library) unique sequences previously unknown for *O.vulgaris*. It is noteworthy to remind that the species has an haploid genome size is estimated to be approximately 5.15 pg (about 5 billion bp; Packard and Albergoni, 1970). The effort here summarized represents a significant but small increase of knowledge related with octopus genome, moreover when it compared to that available for other mollusc such as *Aplysia californica*, which haploid genome size resulted smaller than octopus (for *A. californica* 2.00 pg, Vinogradov, 1998), but which have a large scale of genomic information available (69528 known nucleotide sequences).

However, this initial *in silico* study has allowed the identification of three genes that I utilized for the aim of this thesis: *octopressin*, *cephalotocin* and a short sequence of *stathmin*. Other nucleotidic sequences have been identified following strategies described in Chapter 4.

Table 3.3: This table represents the octopus corenucleotide sequences deposited in GenBank. Among these there are redundant and not redundant sequences.

Organism	Total sequences	Redundant sequences	Not redundant sequences
Octopus aculeatus	3	0	3
Octopus aculifer	2	0	2
Octopus aegina	7	0	7
Octopus alpheus	3	0	3
Octopus aspilosomatis	4	0	4
Octopus aureolatus	4	0	4
Octopus australis	3	0	3
Octopus berrima	8	0	8
Octopus bimaculaides	42	1	41
Octopus bimaculatus	1	0	1
Octopus bocki	1	0	1
Octopus bunurong	3	0	3
Octopus californicus	4	0	4
Octopus conispadiceus	4	0	4
Octopus cyanea	19	2	17
Octopus dofleini	12	0	12
Octopus dyerythraeus	3	0	3
Octopus exannulatus	3	0	3
Octopus graptus	3	0	3
Octopus honkongensis	5	1	4
Octopus incella	1	0	1
Octopus joubini	3	0	3
Octopus kagoshimensis	4	0	4
Octopus kaurna	25	0	25
Octopus laqueus	4	0	4
Octopus longispadiceus	1	0	1
Octopus luteus	1	0	1
Octopus macropus	1	0	1
Octopus magnificus	1	0	1
Octopus maorum	4	0	4
Octopus marginatus	4	0	4
Octopus maya	3	0	3
Octopus mimus	4	0	4
Octopus minor	5	0	5
Octopus mototi	3	0	3
Octopus ocellate	3	0	3
Octopus ocellatus	10	0	10
Octopus oliveri	1	0	1
Octopus ornatus	9	0	9
Octopus pallidus	4	0	4
Octopus parvus	4	0	4
Octopus rubescens	7	0	7
Octopus salutii	2	0	2
Octopus sasakii	3	0	3
Octopus sp.	2	0	2

Organism	Total sequences	Redundant sequences	Not redundant sequences
Octopus sp. 1	1	0	1
Octopus sp. 10-MG-2004	3	0	3
Octopus sp. 2	1	0	1
Octopus sp. 3	1	0	1
Octopus sp. 4	1	0	1
Octopus sp. 5	1	0	1
Octopus sp. 5-MG-2004	3	0	3
Octopus sp. 8-MG-2004	2	0	2
Octopus sp. hakutoensis	2	0	2
Octopus sp. HBH6	1	0	1
Octopus sp. HBH-7	1	0	1
Octopus sp. HBH-B	1	0	1
Octopus sp. NSMT-MOctopus75218	1	0	1
Octopus sp. OM853	3	0	3
Octopus sp. OM870	3	0	3
Octopus sp. OM949	3	0	3
Octopus sp. TL-2006	4	0	4
Octopus sp. xSA-MG-2004	3	0	3
Octopus spring nitryfying crenarchaeote	1	0	1
Octopus tehuelchus	1	0	1
Octopus tetricus	13	2	11
Octopus variabilis	2	0	2
Octopus wolfi	2	0	2
Octopus vulgaris	176	42	134

Table 3.4: This table represents the sepia corenucleotide sequences deposited in GenBank. Among these there are redundant and not redundant sequences.

Organism	Total sequences	Redundant sequences	Not redundant sequences
Sepia aculeata	5	1	4
Sepia apama	39	0	39
Sepia bertheloti	4	0	4
Sepia elegans	13	1	12
Sepia elliptica	3	0	3
Sepia esculenta	24	0	24
Sepia filibrachia	1	0	1
Sepia furcata	4	1	3
Sepia hierredda	5	0	5
Sepia hirunda	4	0	4
Sepia kobiensis	3	0	3
Sepia latimanus	11	0	11
Sepia lorigera	3	0	3
Sepia lycidas	3	0	3
Sepia madokai	1	0	1
Sepia novaehollandiae	1	0	1
Sepia officinalis	329	167	162
Sepia opipara	6	0	6
Sepia orbignyana	3	0	3
Sepia papuensis	3	0	3
Sepia pardex	3	0	3
Sepia peterseni	3	0	3
Sepia pharaonis	42	13	29
Sepia plangon	1	0	1
Sepia recurvirostra	3	0	3
Sepia rex	1	0	1
Sepia robsoni	2	0	2
Sepia smithi	4	0	4
Sepia sp. SI0604	3	0	3
Sepia whitleyana	2	0	2

Table 3.5 This table represents the loligo corenucleotide sequences deposited in GenBank. Among these there are redundant and not redundant sequences.

Organism	Total sequences	Redundant sequences	Not redundant sequences
Loligo bleekeri	67	4	63
Loligo chinensis	5	0	5
Loligo duvauceli	1	0	1
Loligo edulis	3	0	3
Loligo forbesi	39	0	39
Loligo formosana	4	0	4
Loligo gahi	15	6	9
Loligo opalescens	97	4	93
Loligo patagonica	1	0	1
Loligo pealei	122	0	122
Loligo plei	25	0	25

Organism	Total sequences	Redundant sequences	Not redundant sequences
Loligo reynaudii	14	0	14
Loligo sp .AL9407	1	0	1
Loligo sp.	1	0	1
Loligo vulgaris	18	0	18

Table 3.6 This table represents the eledone corenucleotide sequences deposited in GenBank. Among these there are redundant and not redundant sequences

Organism	Total sequences	Redundant sequences	Not redundant sequences
Eledone aldrovandi	2	0	2
Eledone cirrhosa	19	3	16
Eledone massyae	1	0	1
Eledone moschata	2	1	1
Eledone palari	1	0	1

Table 3.7: This table represents the euprymna corenucleotide sequences deposited in GenBank. Among these there are redundant and not redundant sequences

Organism	Total sequences	Redundant sequences	Not redundant sequences
Euprymna berryi	6	0	6
Euprymna hyllebergi	15	0	15
Euprymna morsei	9	0	9
Euprymna scolopes	156	6	150
Euprymna sp.	2	0	2
Euprymna stenodactyla	1	0	1
Euprymna tasmanica	28	0	28

Table 3.8 This table represents the sepiola corenucleotide sequences deposited in GenBank. Among these there are redundant and not redundant sequences

Organism	Total sequences	Redundant sequences	Not redundant sequences
Sepiola affinis	11	0	11
Sepiola atlantica	6	0	6
Sepiola aurantica	2	0	2
Sepiola birostrata	4	0	4
Sepiola ligulata	6	0	6
Sepiola robusta	6	0	6
Sepiola rondeleti	5	1	4
Sepiola rondoleti	2	0	2
Sepiola sp.	2	0	2
Sepiola sp. JMS-2004	6	0	6

Table 3.9: This table represents the sepioteuthis corenucleotide sequences deposited in GenBank. Among these there are redundant and not redundant sequences

Organism	Total sequences	Redundant sequences	Not redundant sequences
Sepioteothis australis	18	0	18
Sepioteuthis lessoniana	87	20	67
Sepioteuthis sepioidea	2	0	2

CHAPTER 4

GENES OF INTEREST

4.1 A short overview on genes of interest

Decades of studies suggest that different behavioural responses, including learned forms of behavioural plasticity, require the activation of a complex biological machinery. Such a complexity is provided by what seems to be an endless and intricate series of different molecular pathways (see for example: Davidson and Erwin, 2006). However, a general consensus exists in considering that training paradigms inducing the formation of long term memory (LTM) require both mRNA and protein synthesis (for review see for example: Yin and Tully, 1996; Kandel, 2001). A short overview of the available knowledge on the machinery involved in the modulation of behavioural responses including learning and memory recall is provided in the introductory section of this thesis. Here I will briefly focus my attention on the role that the genes I considered of interest for this PhD thesis (*creb* , *TH* , *uch* , *stm* , *dat, CT and OP)* have in modulating behaviour and learning processes in invertebrate and vertebrate organisms (table 4.1).

Vasopressin (*AVP*) is certainly a gene more studied in vertebrates than in invertebrates. In the latter (e.g. *Aplysia californica* and *Lymnea stagnalis*) in fact studies have been performed indicating its involvement in response to general maintainance behaviour. In vertebrates (e.g. *Mus musculus*, *Rattus norvegicus, Microtus ochrogaster, Microtus montanus*) instead it was found to be involved in processes activated in response to fear conditioning, spatial learning and social learning. Moreover, the expression in hippocampus and hypothalamus suggests the potential involvement in learning and memory processes (as reviewed in Jarrard, 1993) and in controlling emotions and the autonomic nervous system (for review see Le Doux, 2003; table 4.1).

As is shown in Table 4.1 cAMP response element binding protein (*creb)*, another target gene for this thesis, has been studied extensively in both invertebrates (e.g. *Lymnea stagnalis* and *Aplysia californica*) and vertebrates (e.g. *Mus musculus* and *Rattus norvegicus*). It seems to be involved in all behavioural paradigms under analysis except that in general mainatinance behaviour and imprinting on which no studies have been conducted. In addition, the

expression of *creb* in the hippocampus of vertebrates in response to different behavioural experiences confirms its involvement in mechanisms of learning and memory.

Similarly to *creb,* dopamine transporter (*dat*) has also been the subject of study for the analysis of the molecular mechanisms activated in the learning process of both vertebrates and invertebrates. As reported in Table 4.1, the studies suggest the involvement of *dat* in all behavioural processes analyzed except in habituation. *dat* is involved in the processes of the mesolimbic, mesocortical and nigrostriatal pathways of vertebrates and then involved in motivation and emotional responses (for review see Bressan and Crippa, 2005).

Similarly to *AVP,* oxytocin has been most extensively studied in vertebrates than invertebrates and studies have attested to the involvement in the processes triggered by general maintaninace behaviour and social learning.

The Tyrosine Hydroxilase (*TH*) gene has been studied extensively in many of the organisms under analysis in Table 4.1 both vertebrates and invertebrates. Its expression changes in response to all behavioural paradigms tested except that the classical conditioning and sensitization. In vertebrates, it is involved in the mesocortical, mesolimbic and nigrostriatal pathways capable of mediating motivational and emotional responses like *dat.*

As shown in Table 4.1, there are not many information on the involvement of stathmin (*stm*) in the processes of learning and memory. It is only known its key role in the processes activated in response to fear conditioning and innate fear in mice.

There is also little information on gene ubiquitin C-terminal hydrolase (*uch*), it has only been studied in *Aplysia* and *Mus musculus* among animals considered in Table 4.1 and it is known to play a role in the formation of long-term memory in response to sensitization and spatial learning.

4.1.1 cAMP response element binding protein (CREB)

Early knowledge about molecules involved in the learning process originates from behavioural and molecular studies carried out on the marine snail, *Aplysia californica.* These studies identified the cAMP response element binding protein (CREB) as a key molecule for the formation of long term memory (LTM; as reviewed in Kandel, 2001). In *Aplysia*, repeated mechanical stimulations to the tail or the siphon[1] of the animal result in a long-lasting increase of synaptic strength (i.e. Long-term Facilitation, LTF) and growth of new synaptic connections in a process that requires both mRNA and protein synthesis (for review see Kandel, 2001). In brief, the train of stimuli corresponds to a train of serotonergic stimulation that induces LTF by increasing the levels of cAMP in the cell. This, in turn, activates PKA (or other kinases such as mitogen-activated protein kinase; MAPK) that, once phosphorylated

1 In alternative multiple applications of serotonin to the bath of the neuronal circuit in the *in vivo* preparation (Montarolo *et al.*, Science 234: 1249 (1986).

activates CREB-dependent transcription of early genes (i.e. ubiquitin hydrolase, C/EBP) whose products are necessary for the maintenance of PKA activity and for the activation of late genes (i.e elongation factor 1α) necessary for the induction and maintenance of LTF (e.g. Bacskai *et al.*, 1993). In sum, many studies carried out on several invertebrate and vertebrate species allowed to estabilished the role of CREB in the formation of long term memory (table 4.1) and suggested that CREB is activated during the consolidation; in addition, its regulatory role on transcription of genes determines the strength of memory in a process that is far to be regulated by a few molecules.

4.1.2 Ubiquitin C-terminal hydrolase

Ubiquitin C-terminal hydrolase (Uch) belongs to the PKA-CREB pathway with a recently established role in the formation of LTM. Uch is a protease responsible for removing the ubiquitin from small peptides or larger substrates with a flexible peptide linking the C-terminal domain (reviewed by Wilkinson, 2000). Ubiquitination is a reversible process that has an important role in the targeting of proteins to proteasomes for degradation to regulate several cellular processes such as cell cycle, cell death and circadian rhythms (reviewed by Di Antonio and Hicke, 2004). Enzymes involved in ubiquitination and in the reverse process (deubiquitination) are considered crucial regulatory proteins like kinases and phosphatases. Evidence from *Aplysia*, *Drosophila* and mice suggest that deubiquitinating enzymes and the ubiquitin-proteasome system are important also for synaptic plasticity, learning and memory (for review see for example Di Antonio and Hicke, 2004; table 4.1). In *Aplysia* ubiquitin C-terminal hydrolase (Ap-Uch) enhances proteasome activity by disassembling polyubiquitin chains allowing the substrate degradation by proteasome (Hedge *et al.*, 1997). Ap-Uch favours the removal of inhibitors facilitating signal transduction cascade, transcription and translation of new proteins required for LTF. One of the identified substrates of Ap-Uch is the regulatory subunit (R) of PKA, that links the catalytic subunits inhibiting the enzymatic activity of PKA. Ap-Uch degrades the R subunit allowing the constitutive activation of PKA (Hedge *et al.*, 1997).

Uchl1$^{-/-}$ mice show deficits in memory after passive avoidance trainings, in exploratory behaviour and in hippocampal synaptic plasticity (Sakurai *et al.*, 2008). Impairment of memory was also associated with alterations in CREB phosphorylation and the subsequent failure in the LTP maintenance in the hippocampus, these findings suggest that UCH-L1 activity is crucial also for the temporal integrity and persistent phosphorylation of CREB required for LTP and for the formation of long term memory.

4.1.3 Stathmin

Learning and memory processes require synaptic plasticity, which implies cytoskeleton dynamics. Stathmin (also known as Oncoprotein 18, Op18), is the most prominent member of a conserved protein family that is involved in the regulation of microtubule dynamics (for review see Steinmetz, 2007). It is a cytosoluble phosphoprotein which destabilizes microtubules by binding tubulin dimers and inhibiting polymerization of tubulin subunits (e.g. Larsson *et al.*, 1999). Microtubule filaments[2] play an essential role in intracellular transport and more generally in cytoskeleton dynamics, known to be involved in a variety of biological processes.

Many experimental studies suggest that stathmin, as a modulator of microtubule dynamics, is implicated in neural plasticity, learning and memory processes (reviewed in Mori and Morii, 2002; e.g. Nelson *et al.*, 2004; Hayashi *et al.*, 2006; Kruger *et al.* 2006; table 4.1).

Behavioural studies conducted on *stathmin* knockout mice show that these mutants appear deficient in conditioned fear responses, suggesting that *stathmin* is essential in regulating both innate and learned fear. Furthermore, experiments carried out on brain slice preparations from *stathmin* knockout mice show difficulties in inducing normal LTP, thus suggesting that *stathmin* is involved also in the regulation of LTP and consequently in synaptic plasticity processes (Shumyatsky *et al.*, 2005).

4.1.4 Tyrosine hydroxylase and dopamine transporter

Several studies in mammalian and invertebrate systems have shown that catecholamines are involved in synaptic modulation and behavioural plasticity (e.g. Riemensperger *et al.*, 2005; Stefani and Moghaddam, 2006), two physiological events which underlie the learning process. Catecholamine neurotransmitters include dopamine, noradrenaline, and adrenaline and act as transmitters in the nervous systems of both vertebrates and invertebrates.

The enzyme tyrosine hydroxylase (TH) catalyzes the first and rate-limiting step in catecholamine biosynthesis (reviewed in Zigmond *et al.*, 1989) and it plays an important role in the regulation of processes activated in response to different behavioural paradigms. In *C. elegans,* animals deficient for *cat-2* (the gene coding for a tyrosine hydroxylase enzyme) show impairement in the regulation of the locomotory rate in response to its food (bacteria). Wild-type well-fed worms move more slowly in presence of bacteria, while *cat-2* mutants are deficient in food sensing, they fail to slow down the locomotor rate. These findings suggest that this gene (*cat-2*) and more in general dopamine seem to mediate slowing locomotor

2 they are dynamic polymers made of α/ β-tubulin heterodimers

response (a rewarding experience) to bacteria (Sawin *et al.*, 2000).

In mice with TH mutation ($TH^{+/-}$) noradrenaline (NA) accumulation in the brain was moderately decreased to 73–80% of the wild-type. Conditioned learning of these mice was tested with an active avoidance paradigm and results indicate that reduced NA metabolism in the brain causes a mild impairment of active avoidance learning.

In DD mice in which TH expression was allowed only in noradrenergic and adrenergic cells, defective dopamine neurotransmission leads to impairment of multiple behavioural functions: spontaneous and drug-induced locomotor activity, active avoidance, instrumental conditioning. and associative learning (for details table 4.1).

Another molecule involved in dopamine metabolism is the dopamine transporter (DAT). DAT as well as norepinephrine transporter (NET) and serotonin transporter (SERT) are members of the Na^+/Cl^- dependent neurotransmitter transporter family (reviewed by Amara and Kuhar, 1993; Mortensen and Amara, 2003). DAT is a membrane symporter that clears DA from the synaptic cleft and serves as an important regulator of signal amplitude and duration at the dopaminergic synapses (reviewed by Gainetdinov and Caron, 2003).

Alteration in the function of DAT results in expected disruption of extra-cellular dopamine clearance and prolonged extra-cellular lifetime of DA. This increase of DA caused disruption of normal locomotor activity such as deficits in several cognitive and behavioural processes in both vertebrate and invertebrate organisms.

Mice knockout (DAT KO) or knockdown (DAT KD) for *Dat* displayed a distinct behavioural phenotype which comprises novelty induced hyperactivity, decreased habituation, locomotor activity dysregulation, lactation and maternal behaviour deficits in the females, impairments in learning and memory of place preference and instrumental conditioning tasks (reviewed by Gainetdinov and Caron, 2003). Moreover the mutant mice demonstrated abnormal social interaction, they displayed more reactive and more aggressive behaviours promoting social isolation or a complete inversion of social hierarchy.

Rats subjected to pharmacological treatments (*i.e.* cocaine, RTI-336) inhibiting the DAT function showed that increased inhibition of DAT activity contributed to locomotor sensitization and deficits in some cognitive processes induced in response to conditioned taste aversion, instrumental conditioning and conditional place preference tasks (table 4.1).

4.1.5 Cephalotocin and octopressin

Vasopressin (AVP) and oxytocin (OXT) are neuropeptides that have profound effects on a variety of mnemonic and social processes (e.g. social recognition memory, olfactory recognition memory and in partner and offspring bonding as well as in aspects of social trust and anxiety; as reviewed in Gulpinar and Yegen, 2004; Sanchez-Andrade and Kendrick,

2009). AVP and OXT are able to influence "social memory" that is a unique form of memory, critical for reproduction, territorial defence, and the establishment of dominance hierarchies in nature. These molecules influence social behaviour including affiliation, male courtship, aggression, and reproduction in a wide range of taxa (Trainor *et al.*, 2003; Toyoda *et al.*, 2003; Goodson and Bass, 2000; Castagna *et al.*, 1998; Goodson *et al.*, 2004; Levoye *et al.*, 2005; Kawada *et al.*, 2004; table 4.1).

AVP also plays an important role in several types of learned behaviours such as active or passive avoidance conditioning, aggressive behaviour and appetite conditioning (as reviewed in Keverne, 2004). On the other hand, OXT coordinates maternal behaviour and physiology to ensure successful maternal care. This neuropeptide is also involved in the control of social recognition and in olfactory memory, two important components of maternal care (Pedersen and Prange, 1979).

The members of the AVP/OXT superfamily are widely distributed throughout the animal kingdom: vertebrates, arthropods, annelids and mollusks (Cruz *et al.*, 1987; Proux *et al.*, 1987; Reich, 1992; Salzet *et al.*, 1993; Oumi *et al.*, 1994; Vankesteren *et al.*, 1995). All vertebrate species except for cyclostomes contain at least one VP and one OXT family member (Heierhorst *et al.*, 1992). In invertebrates, VPlike diuretic hormone is the unique family member present in the locust *Locusta migratoria*, annetocin in the earthworm *Eisenia foetida*, Lys- and Arg-conopressins in the gastropod molluscs *Conus geographus*, *Conus striatus* and *Lymnaea stagnalis* (reviewed by Hoyle, 1998).

Octopus vulgaris, similarly to what occurs in vertebrates, possesses two members of the oxytocin/vasopressin superfamily: octopressin (OP) and cephalotocin (CT). This was the first observation of the co-occurrence of two members of the superfamily in an invertebrate species (Kanda *et al.*, 2003).

In situ hybridization histochemistry analysis conducted on OP mRNA demonstrates that it is expressed in many lobes of the octopus brain. The expression of OP mRNA in the superior and posterior buccal lobes suggests that OP may play important roles in feeding behaviour. On the contrary, its expression in the vertical, subvertical, superior and inferior frontal lobes known to form a series of matrices concerned with touch and visual learning suggest that OP may contribute to some roles in the memory and learning system of octopus. The presence of OP mRNA in other lobes of supraesophageal mass (i.e. anterior, medial and dorsal basal lobes) suggest that it may be a multifunctional neuropeptide probably contributing to walking, swimming, changing of color, respiration, escape jetting, etc. (Takuwa-Kuroda *et al.*, 2003).

Moreover the OP and OP receptor (OPR) are distributed also in the peripheral tissues where they are involved in the tonus control of the rectum, oviduct, brachial vessel, spermatophoric gland and contraction of ring slice of the anterior aorta (Kanda *et al.*, 2005).

CT is expressed in neurons in the ventral median vasomotor lobe. Some of these send out axons and form an extensive neuropile inside the vena cava in direct contact with the circulating blood, making a neurosecretory system (Takuwa-Kuroda *et al.*, 2003). Some experiments studying the expression of CT receptor (CTR) in the octopus brain suggest the involvement of CT in neurotransmission and neuromodulation. CTR mRNA has been found in the optic lobe, in the olfactory lobe and in the peduncle lobe suggesting the role of CT in sensory systems such as vision and olfaction. Moreover the CTR expression in the buccal ganglion and the gastric ganglion may suggest a contribution of CT to feeding behaviour.
In addiction, the CT seems to play a role also in the maturation and encapsulation of eggs in the octopus oviduct (Kanda *et al.*, 2003)

Table 4.1 – A tabularized overview of the studies testing the involvement of CREB, dat, TH, stathmin and uch in processes of learning and memory formation. In the table, the organisms and the behavioural paradigms tested are reported together with the brain areas (Arabic numbers) where target gene expression has been studied, when this information is available (1: amygdala; 2: anterior dierdic neurons; 3: area X; 4: buccal ganglia, 5: cephalic neurons, 6: cerebral ganglia; 7: hippocampus; 8: hypothalamus; 9: locus coeruleus; 10: medial gianiculate nucleus, 11: medial prefrontal cortex; 12: mushroom bodies; 13: nucleus accumbens; 14: olfactory bulb; 15: par-olfactory lobe; 16: pedal ganglia; 17: posterior intralaminar nuleus; 18: sensory neurons; 19: supraoptic nuclei; 20: striatum; 21: substantia nigra compacta; 22: ventral tegmental area; √: no data about brain region).

		General maintainance behaviour							Habituation							Sensitization							Classical conditioning							Fear conditioning							Operant conditioning							Spatial learning							Imprinting							Social learning (including bird song learning)						
		AVP	CREB	dat	OXT	TH	stm	uch	AVP	CREB	dat	OXT	TH	stm	uch	AVP	CREB	dat	OXT	TH	stm	uch	AVP	CREB	dat	OXT	TH	stm	uch	AVP	CREB	dat	OXT	TH	stm	uch	AVP	CREB	dat	OXT	TH	stm	uch	AVP	CREB	dat	OXT	TH	stm	uch	AVP	CREB	dat	OXT	TH	stm	uch
Caenorhabditis elegans				2 5		√							√																																															
Eisenia foetida					√																																																						
Lymnea stagnalis		6 16																4 6													√																												
Aplysia californica		√									18							18																																									
Drosophila melanogaster		√			√						12														√						√ √																												
Taeniopygia guttata																																																											
Microtus ochrogaster																																																											
Microtus montanus																																																											
Mus musculus		13 20 21				√				√ 14 20			9 14																		1 7 10 17				√	1	√				7 13 13 20				20 21				7 20 20 21																		
Rattus norvegicus		13 20 21 22																															√					√ 7	√		21									8 7		19	11																

This table have been prepared on the basis of data included in the following works (hereunder arranged according to the animal species). The behavioural paradigms are reported as reviewed by Moore (2004) with the exception of the category of general maitainance learning identified by Grier and Burk (1992).

Aplysia californica (Martinezpadron and Lukowiak, 1992; Kaang *et al.*, 1993; Bartsch *et al.*, 1998; Silva *et al.*, 1998; Kandel, 2001; Josselyn and Nguyen, 2005; Lee *et al.*, 2008)

Caenorhabditis elegans (Sawin *et al.*, 2000; Sanyal *et al.*, 2004; McDonald *et al.*, 2007; Carvelli *et al.*,. 2008)

Drosophila melanogaster (Levin *et al.*, 1992; Yin *et al.*, 1994; Tully *et al.*, 1994; Yin *et al.*, 1995; Silva *et al.*, 1998; Neckameyer, 1998; Neckameyer *et al.*, 2000; Pendleton *et al.*, 2002; Tully *et al.*, 2003; Kume *et al.*, 2005; Neckameyer and Weinstein, 2005; Pendleton *et al.*, 2005; Zhang *et al.*, 2008; Liu *et al.*, 2008)

Eisenia foetida (Oumi *et al.*, 1996)

Lymnea stagnalis (Vankesteren *et al.*, 1995; Ribeiro *et al.*, 2003; Azami *et al.*, 2006)

Microtus ochrogaster (Young, 2002)

Microtus montanus (Young, 2002)

Mus musculus (Impey *et al.*, 1998; Silva *et al.* 1998; Nishii *et al.* 1998; Kobayashi and Sano, 2000; Kobayashi *et al.* 2000; Gainetdinov *et al.*, 2001; Filipenko *et al.*, 2001; Kobayashi and Kobayashi, 2001; Kida *et al.*, 2002; Shumyatsky *et al.*, 2002; Young, 2002; Bozon *et al.*, 2003; Fernagut *et al.*, 2003; Josselyn *et al.*, 2004; Brodie *et al.*, 2004; Morice *et al.*, 2004; Hironaka *et al.*, 2004; Rodriguiz *et al.*, 2004; Winslow and Insel, 2004; Walters *et al.*, 2005; Shumyatsky *et al.*, 2005; Medvedev *et al.*, 2005; Wood *et al.*, 2005; Chen *et al.*, 2006; Tillerson *et al.*, 2006; Cagniard *et al.*, 2006; Yin *et al.*, 2006; Martel *et al.*, 2007; Sindreu *et al.*, 2007; Tilley *et al.*, 2007; Morice *et al.*, 2007; Weiss *et al.*, 2007; Russell, 2007; Robinson *et al.*, 2007; Porte *et al.*, 2008; Han *et al.*, 2008; Tropea *et al.*, 2008; Perona *et al.*, 2008; Niimi *et al.*, 2008; Palmiter, 2008; Peace *et al.*, 2008; Sakurai *et al.*, 2008).

Rattus norvegicus (Guzowski and McGaugh, 1997; Lamprecht *et al.*, 1997; Izquierdo *et al.*, 2000; Cammarota *et al.*, 2000; Viola *et al.*, 2000; Josselyn *et al.*, 2001; Diaz-Veliz *et al.*, 2002; Zahniser and Sorkin, 2004; Gulpinar and Yegen, 2004; Keverne and Curley, 2004; Freeman *et al.*, 2005; Faure *et al.*, 2005; Lindblom *et al.*, 2006; Tillerson *et al.*, 2006; Carroll *et al.*, 2006; Clinton *et al.*, 2006; Engelmann *et al.*, 2006; Hubbard *et al.*, 2007; Flagel *et al.*, 2007; McDougall *et al.*, 2008; Todeschin *et al.*, 2009).

Taeniopygia guttata (Soha *et al.*, 1996; Sasaki *et al.*, 2006).

4.2 Octopus gene sequences identification: Materials and methods

An aliquot of cDNA library or alternatively an aliquot of cDNA synthesized by Invitrogen Kit (for details see paragraph 7.2.4) was used as the template of the PCR reaction (see paragraph 4.2.1). Each PCR reaction was performed with primers specific for target genes (table 4.2). The amplified fragment was visualized by electrophoretic analysis on agarose gel, eluted from the gel and cloned into the TOPO TA PCRII vector (Invitrogen) and used to transform bacterial. Positive clones were selected by PCR screening and used for plasmid DNA purification. The purified plasmid containing the target cDNA fragment was sequenced by the Molecular Biology Service of the Stazione Zoologica "Anton Dohrn" in Naples using the Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). The obtained sequences were translated and analyzed for homology as summarized below.

4.2.1 PCR

Polymerase chain reaction (PCR) is a method that allows logarithmic amplification of short DNA sequences. Each amplification reaction was conducted in a volume of 50 µl containing: 100 ng DNA template or 2 µl of an aliquot of cDNA library, 1X synthesis buffer (50 mM potassium chloride, 10 mM Tris-HCl pH 8.3, 1.5 mM magnesium chloride), 0.2 mM dNTP mix, 50 pmol/µl of each primer (table 4.2), 1U/µl Taq DNA polymerase and sterile H_2O. The amplification cycles were conducted by Peltier Thermal Cycler PTC-200 (MJ Research). After denaturation at 95 °C for five minutes, 25-35 amplification cycles were structured as follows: denaturation at 94 °C for 30 seconds, annealing at 55-65 °C for 30 seconds, extension at 72 °C for 0.5 - 3 minutes (considering 1 minute to synthesize 1 kb fragment). Finally, an extension cycle was carried out at 72 °C for 10 minutes to complete all the strands.

Table 4.2 : Primer sequences, amplicon size and template used in PCR reaction to find octopus gene sequence.

Gene	GenBank accession number	Primer	Primer sequence 5' - 3'	Amplicon size	Template for PCR reaction
Ov - dat	FJ617441	F	tckggiaargtdgtbtggtt		
		R	atigcycigadccnccraa	506	octopus brain cDNA
		T7 F	gcgcgcgtaatacgactcactatag		octopus brain cDNA
		R	atctgacaccagacttctc	1113	library
Ov - stmn	GQ152874	F	tggagagaaaaggccaaaga		
		R	caatagcctcctgggtgaga	396	octopus brain cDNA

Gene	GenBank accession number	Primer	Primer sequence 5' - 3'	Amplicon size	Template for PCR reaction
Ov - TH	FJ617442	F	rtsttycagwsyacicagta		
		R	aaytcvacrgtgaaccagta	558	octopus brain cDNA
		T3 F	gcgcaattaaccctcactaaaggga		octopus brain cDNA library
		R	actcagcgaattcaggatc	1199	
Ov - uch	GQ148556	F	tggmivscwitkgaatcmaa		
		R	gcmaiwgticcrcadgcrtt	254	octopus brain cDNA
		F	tcaaaccccgaggttctaaat		
		R	cgattgtttgacggatgaaa	224	octopus brain cDNA

4.2.2 DNA gel electrophoresis and extraction

To check the length of DNA from enzymatic digestions or PCR amplifications, DNA samples containing 1X Gel Loading buffer[3] were run in agarose gel[4] in 1 X TAE buffer[5]. QIAquick Gel Extraction Kit (QIAGEN) was used to extract and purify DNA of 70 bp to 10 kb from agarose gels and for DNA cleanup from enzymatic reactions following manufacturers' instructions.

4.2.3 Cloning reaction of PCR product

PCR products were cloned in TOPO TA PCRII vector (Invitrogen) following the manufacturers instructions. Briefly a mix of 6 µl was composed with TOPO vector[6], salt solution[7], 3 -5 folds vector moles of PCR product and sterile water. The samples were mixed gently and incubated for 5 minutes at room temperature. Then they were placed on wet ice prior to cell transformation.

4.2.4 Chemical transformation and bacterial electroporation

The circular plasmid DNA and competent bacterial cells (Molecular Biology Service at the Stazione Zoologica Anton Dohrn) were placed on ice for 15 minutes. For the chemical transformation, cells were incubated for one minute at 42 °C and another minute on ice. For electroporation the cells were placed in a cold electrocuvette. The electrocuvette was subjected to an electric pulse at constant 1.7 V using a Bio-Rad

3 Gel Loading buffer 6X: 0.25% (w/v) Bromophenol Blue; 15% (v/v) Ficoll 400; 120 mM EDTA; and 0.25% (w/v) Xylene Cyanol FF)

4 Agarose gel 1% to 2% containing 0.5 µg/ml of Ethidium Bromide

5 TAE buffer 1X: 0.04 M Tris-Acetate; 0.001 M EDTA pH 8.0

6 containing 10 ng/µl of plasmid DNA, 50% glycerol, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM DTT, 0.1% Triton X-100, 100 µg/ml BSA, 30 µM bromophenol blue

7 Salt solution: 1.2 M NaCl, 0.06 M $MgCl_2$

Gene Pulser electroporation apparatus. Then the cells, transformed in any of the either methods (chemical transformation or electroporation) were added to 250 µl of SOC[8] and shaken at 270 rpm at 37 °C for 1 hour. Then they were plated on LB solid medium[9] in the presence of the specific antibiotic (50 µg/ml) to which the plasmid is resistant, and then grown at 37°C overnight.

4.2.5 Colony PCR

It is possible to carry out a PCR reaction using as template the DNA of a single bacterial colony while the colony is growing. Half of each single colony was placed in a PCR mixture described previously (for details see paragraph 4.2.1) and half was grown in 3 ml of LB liquid medium in the presence of the suitable antibiotic (50µg/ml) shaking at 37 °C for 8-12 hours. The PCR reaction had the following composition: 1X PCR buffer (Boehringer Manneheim, Monza, Italy); 0.2 mM dNTP mix; 1 pmol/µl of each primer (forward and reverse); and 0.5 U/µl Taq DNA polymerase. PCRs were carried out with the following protocol: denaturation at 95 °C for 30 seconds, annealing at 55-65 °C for 30 seconds, extension at 72 °C for 1-2 minutes. By electrophoresis analysis, the samples presenting a band of expected size were identified and plasmid DNA was purified from the corresponding bacterial colonies.

4.2.6 DNA mini preparation

A single bacterial colony containing the plasmid DNA of interest was grown in a suitable volume of LB medium in the presence of the appropriate antibiotic shaking at 37 °C overnight. For preparation of up to 20 µg of high-copy plasmid DNA, the colony was placed in 3 ml of LB medium and the QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden) was used for plasmid DNA purification following the manufacturer instructions. Briefly, an alkaline lysis buffer was used to lysate bacterial cells. The lysate is subsequently neutralized and adjusted to high-salt binding conditions in one step. After lysate clearing, the sample is ready for purification on the QIAprep silica membrane that permits a selective adsorption of plasmid DNA in high-salt buffer and elution in low-salt buffer.

8 SOC: tryptone 20 g/l, yeast extract 5 g/l, 10 mM NaCl, 2.5 mM KCl, 100 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose

9 LB solid medium: NaCl 10 g/l, bacteotryptone 10 g/l, yeast extract 5 g/l, agar 15 g/l

4.2.7 DNA maxi preparation

For preparation of up to 500 μg of high-copy plasmid DNA, a single colony from a freshly streaked plate was inoculate into 500 ml of LB medium. The QIAGEN plasmid maxi kit was used to purify the plasmid DNA according to the manufacturer protocol. The bacterial cells were lysated in an alkaline lysis buffer. Then the plasmid DNA was separated by other molecules using an anion-exchange resin column under appropriate low-salt and pH conditions. RNA, protein and low-molecular weight impurities were removed by a medium-salt washes. The plasmid DNA was eluted in a high-salt buffer, then concentrated and desalted by isopropanol precipitation.

4.2.8 Protein sequence alignment and study of homology

The new identified octopus genes sequence was translated and aligned with orthologous sequences of invertebrate and vertebrae organisms using ClustalX2 software (Thompson *et al.*, 1997).
Then the identity score of sequences was calculated using another software: GeneDoc which allows to calculate the percentage of sequence residues that are identical in every pair of sequences in the alignment. Such identity tables have been used as a way to describe the degree of divergence among the sequences in the alignment.

4.3 Octopus gene sequences identification: Results and discussion

4.3.1Octopus vulgaris *creb*

The *O. vulgaris* c-AMP response element binding (*Ov-creb*) cDNA sequence was found after the screening of an *O. vulgaris* cDNA library constructed from mRNA extracted from the supra-oesophageal mass as described in Sirakov *et al.* (2009). A sequence of 4313 bp was identified that codes for a protein of 295 amminoacids (A. Arcucci, unpublished data). Comparison of amminoacid sequences deduced from *Ov-creb* with CREB proteins of other species confirms the evidence of high conservation of the most important functional domains [such as the kinase-inducible domain (KID) and leucine-zipper bZIP domain, figure 4.1] in vertebrate and invertebrate organisms.
The KID domain contains the serine residue by which phosphorylation allows the activation of CREB. Moreover this domain contains multiple potential phosphorylation sites for various protein kinases that regulate CREB activity and specificity (Shaywitz and Greenberg, 1999).

The KID domain of CREB plays an important role not only for activation of CREB but also for CREB-mediated transcription processes (Sun and Maurer, 1995; Cha-Molstad *et al.*, 2004). In fact, it is important for the interaction with the KIX domain of the CREB binding protein (CBP) that represents a molecular bridge between upstream transcription factors and the RNA polymerase II transcription complex (Struhl, 1998; Cho *et al.*, 1998).

Another highly conserved region of the CREB protein is the bZIP domain. This domain is a structural motif at the carboxyl-terminal of the protein formed by a heptad repeat of leucine residues (Dwarki *et al.*, 1990). Presence of both the basic and leucine zipper (bZIP) domains places CREB within a larger family of bZIP transcription factors, including mammalian c-Fos, c-Jun, c-Myc, and C/EBP, as well as yeast Gcn4 (Vinson *et al.*, 1989; Struhl, 1989). DNA binding is mediated by a basic domain, a lysine- and arginine-rich stretch of amino acids located just amino-terminal to the leucine zipper (Dwarki et al. 1990). CREB binds to its DNA target sequence as a dimer (Yamamoto *et al.*, 1988) and the dimerization process is mediated principally by the bZIP domain.

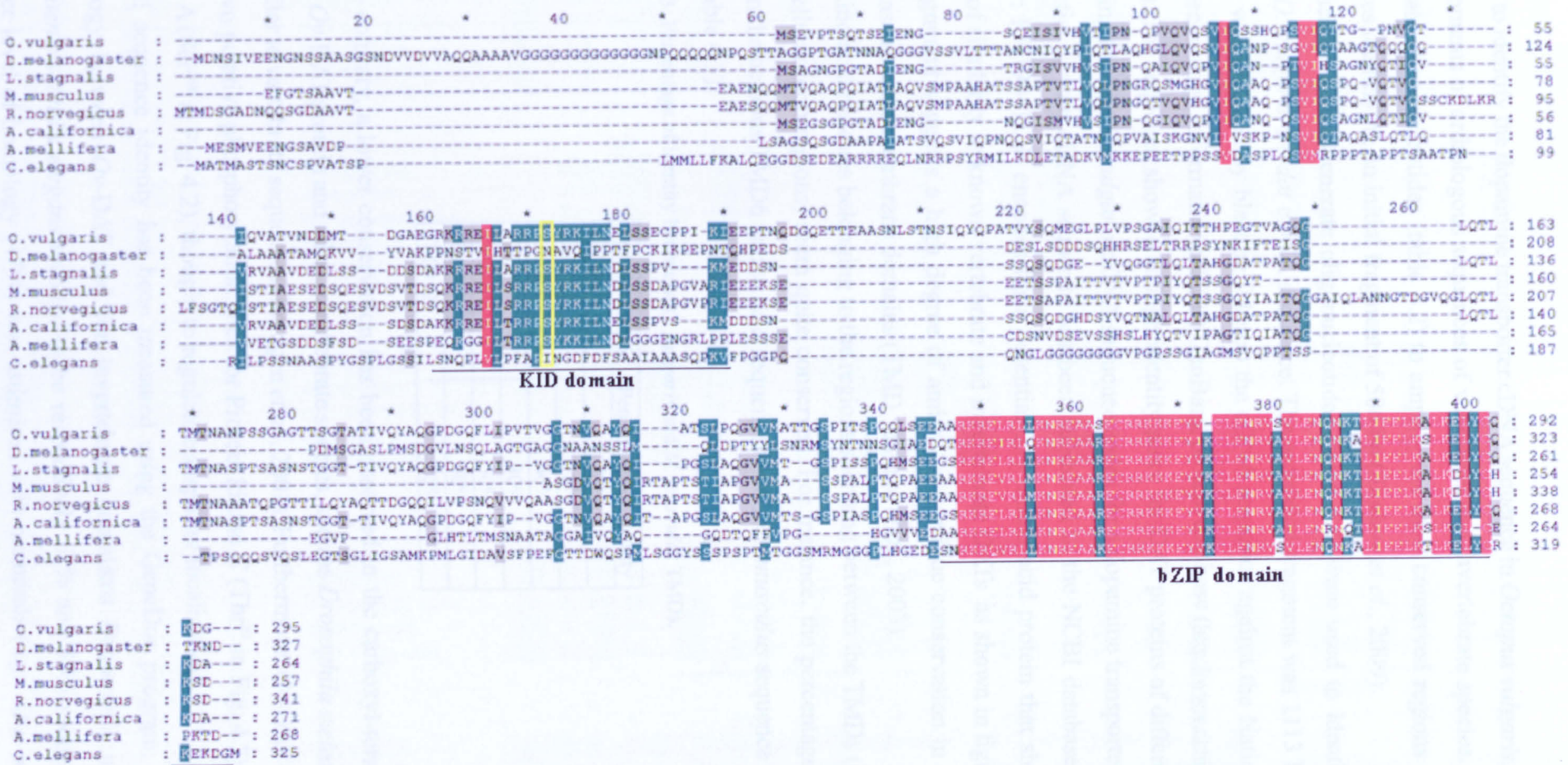
An overall analysis of alignment of CREB sequences, as reported in figure 4.1, allows the estimation of sequence homology between *Ov*-CREB and other vertebrate and invertebrate organisms.

Amminoacid homology is particularly striking between *Octopus vulgaris* CREB and the other mollusks (*A. californica* and *L. stagnalis*) demonstrating a 58% sequence identity.

Sequence homology is weaker, but still apparent in particular in the most conserved regions, between *Octopus vulgaris* CREB and mammals (*M. musculus* and *R. norvegicus*) with 33% identity.

Inside the KID domain, the position of the serine residue, which is phosphorylated by PKA to activate CREB, shows high conservation among the analyzed sequences. Whereas in the KID domain the position of the serine residue phosphorylated by Ca^{2+}/calmodulin-dependent kinase (CaMK) appears conserved in all investigated sequences except for *Apis* CREB, where the phospho-serine is closest to the carboxyl-terminal extremity (Eisenhardt *et al.*, 2003).

Figure 4.1 – This figure shows the sequence comparison between CREB sequences from *Octopus vulgaris* (Accession number: FJ617443), *Drosophila melanogaster* (NP_001097017), *Lymnea stagnalis* (BAC20140), *Mus musculus* (AAB64015), *Rattus norvegicus* (P15337), *Aplysia californica* (Bartsch *et al.*, 1998), *Apis mellifera* (CAD24872), *Caenorhabditis elegans* (NP_001022859). Phosphorylation sites for cAMP-dependent protein kinase (PKA) are marked with a yellow square and for CamK with blue one. A black line is used to underline the most conserved regions of the KID and bZIP domains. The amminoacid residues with 100% identity are reported in red, with 80% identity in green and 60% in grey.


KID domain
bZIP domain

4.3.2 Octopus vulgaris dopamine transporter

In order to identify the dopamine transporter cDNA sequence in *Octopus vulgaris*, I analyzed the alignments of orthologous sequences of vertebrate and invertebrate species. I designed degenerate oligonucleotides (table 4.2) to amplify the most conserved regions of peptidic sequences identifying an initial fragment of 506 bp (Sirakov *et al.*, 2009).

Subsequently, non-degenerate oligonucleotides (table 4.2) were used to identify another part of *O. vulgaris Ov-dat* cDNA sequence. The amplified fragment was 1113 bp long. Its identity was revealed by blasting (blastx) the cDNA sequence against the National Centre for Biotechnology Information (NCBI) GenBank protein database (http://www.ncbi.nlm.nih.gov/BLAST/). The sequence showed an high identity score for DAT proteins of different species, suggesting that the *O. vulgaris* cDNA sequence codes for a dopamine transporter.

The partial *Ov-dat* cDNA sequence has been submitted to the NCBI database (accession number: FJ617441). It encodes for a potential 330 amino acid protein that shows a high degree of similarity to known vertebrate and invertebrate DATs as shown in figure 4.2.

The alignment suggests a high degree of amino acid residue conservation in the regions known as the trans-membrane domains (TMD, Gallant *et al.*, 2003).

The amino acid residues belonging to the regions spanning between the TMDs (cytosolic or extra-cellular loop regions) seem quite conserved. For instance, the percentage of identity between the octopus TMD6 amino acid sequences and *Mus musculus* sequence is generally high (table 4.3).

Table 4.3: Percentage of identity beteween *O. vulgaris* and *M. musculus* TMDs.

Protein domain	Percentage of identity
TMD6	78%
TMD7	58%
TMD8	66%
TMD9	30%
TMD10	50%
TMD11	40%
TMD12	35%

On the contrary, a lower conservation has been observed in the carboxyl-terminal region where *Ov*-DAT protein and other invertebrate species, such as *Drosophila melanogaster,* are dissimilar to vertebrate sequences (Porzgen *et al.*, 2001). Furthermore, *Ov*-DAT loop regions hold two putative phosphorylation sites for Protein kinase C (Thr^{87} in Fig. 4.2) and Protein kinase A (Ser^{208} in Fig. 4.2), thought to regulate transporter localization.

Overall sequence identity has been measured using the GeneDoc program. Amino acid homology between *Ov*-DAT and the invertebrate organisms *Drosophila melanogaster* and *Caenorhabtidis elegans* resulted to be respectively 61% and 50% sequence identity. A lower level of homology (47%) was estimated with mammals (e.g.: *Mus musculus* and

Rattus norvegicus) that appeared dissimilar from the invertebrates sequences particularly in C-terminal region.

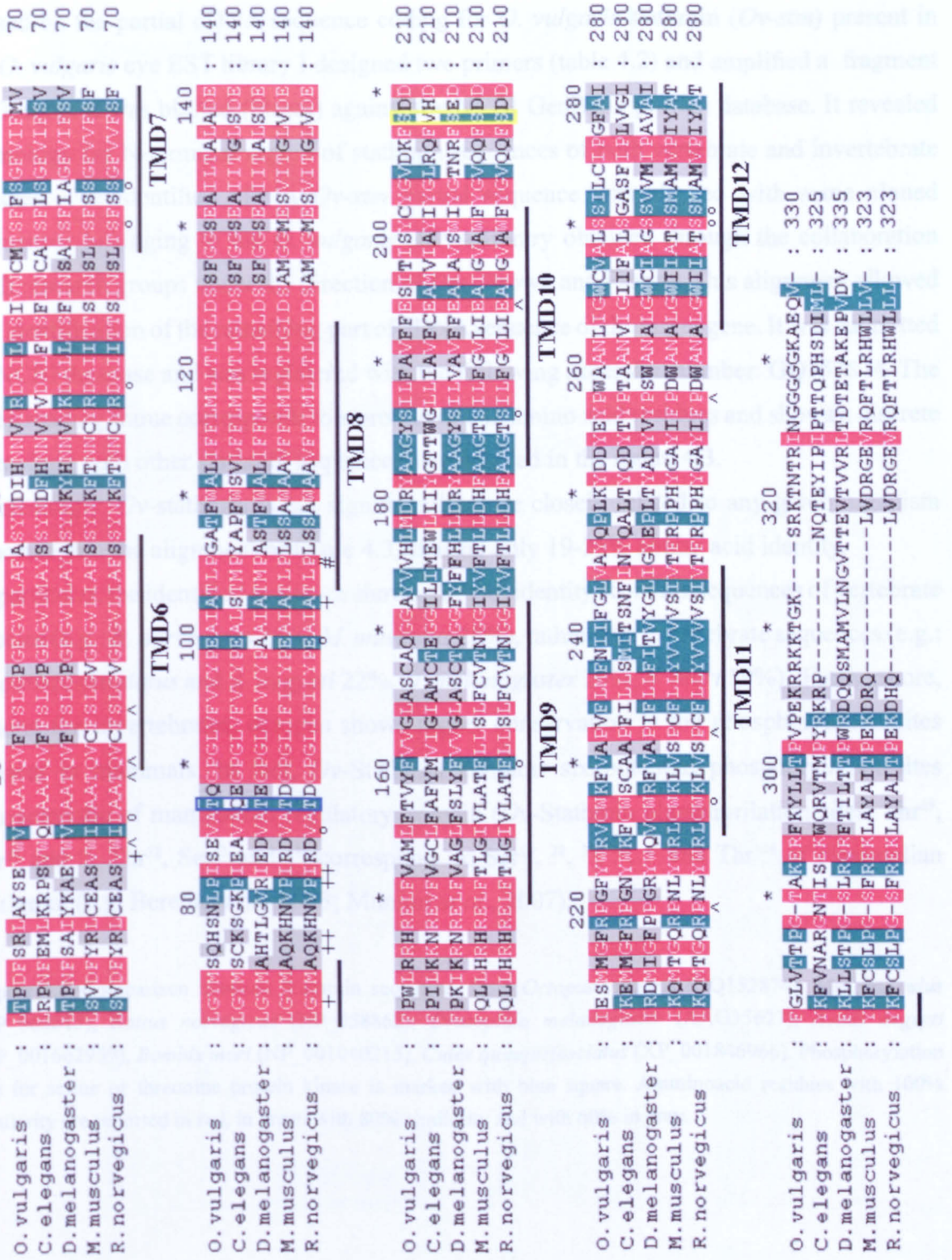


Figure 4.2 –The comparison between DAT sequences from *Octopus vulgaris* (FJ617441) *Caenorhaditis elegans* (Q03614), *Drosophila melanogaster* (AAF76882), *Mus musculus* (AAB64015) and *Rattus norvegicus* (P23977). Phosphorylation site for Protein kinase C (PKC) is marked with blue square, whereas for Protein Kinase A (PKA) with a yellow one. Black lines are used to highlight 7 of 12 characteristic trans-membrane domains (TMD)(e.g. Gallant *et al.*, 2003). Aminoacid residues with 100% similarity are reported in red, in green with 80% similarity and with 60% in grey. As reviewed by Voltz & Schenk (2005) amino acid residues known to be involved in maintenance of DAT activity (+), in DA uptake (°), in the control of DA uptake and DAT plasma membrane expression (^) have been identified in humans and rats.

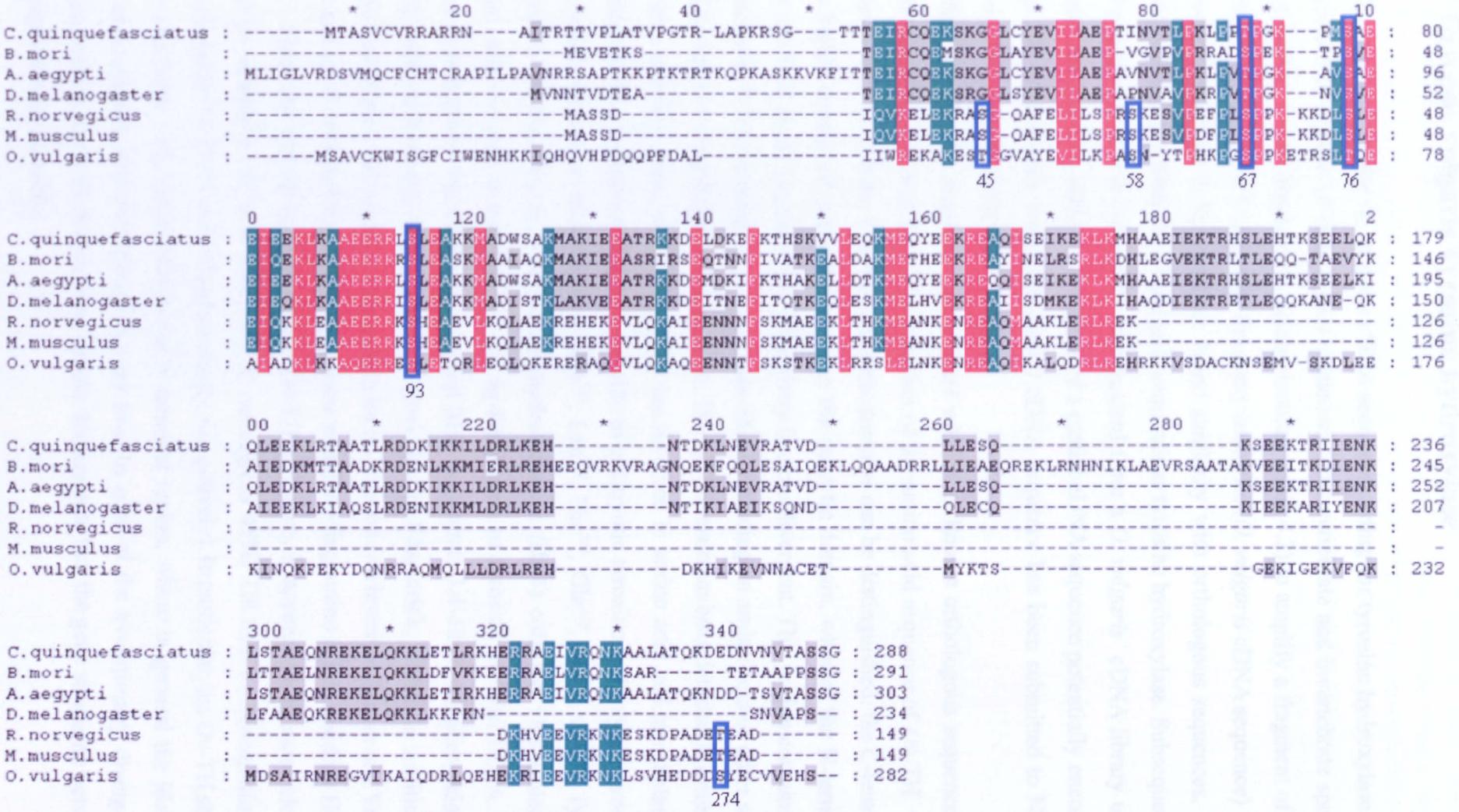
4.3.3 Octopus vulgaris stathmin

Based on the partial cDNA sequence coding for *O. vulgaris* stathmin (*Ov-stm*) present in an *O. vulgaris* eye EST library I designed two primers (table 4.2) and amplified a fragment of 396 bp, it was blasted (blastx) against the NCBI GenBank protein database. It revealed homology for N-terminal region of stathmin sequences of both vertebrate and invertebrate species. The identified cDNA *Ov-stm* partial sequence was aligned with some cloned sequences belonging to the *O. vulgaris* cDNA library obtained through the collaboration with research groups under the direction of Drs. Brown and Fiorito. This alignment allowed the identification of the remaining part of cDNA sequence of the target gene. It was submitted to NCBI database and was registered with the following accession number: GQ152874. The open reading frame corresponds to a protein of 282 amino acid residues and shows a discrete homology with other stathmin sequences as displayed in the figure 4.3.

Interestingly, *Ov*-stathmin is not significantly more closely related to any given organism reported into the alignment of figure 4.3, sharing only 19-25% amino acid identity.

In particular, the identified sequence shows a higher identity score for sequences of vertebrate species (e.g.: *R. norvegicus* 25%, *M. musculus* 25%), rather than invertebrate sequences (e.g.: C. *quinquefasciatus* and *A. aegypti* 22%, *D. melanogaster* 20%, *B. mori* 19%). Furthermore, among the invertebrates, octopus shows higher conservation to the phosphorylation sites present in mammals. In fact, *Ov*-Stathmin contains six potential phosphorylation sites characteristic of mammalian regulatory domain (*Ov*-Stathmin phosphorilation sites: Thr^{45}, Ser^{58} and ^{67}, Thr^{72}, Ser^{93} and ^{274} correspond to Ser^{16}, ^{28}, ^{38}, ^{46}, ^{63} and Thr^{146} of mammalian stathmin; (e.g. Beretta *et al.*, 1993; Munton *et al.*, 2007).

Figure 4.3–Comparison between Stathmin sequences from *Octopus vulgaris* (GQ152874), *Mus musculus* (NP_062615), *Rattus norvegicus* (NP_058862), *Drosophila melanogaster* (AAG35627), *Aedes aegypti* (XP_001662959), *Bombix mori* (NP_001040215), *Culex quinquefasciatus* (XP_001846966). Phosphorylation site for serine or threonine protein kinase is marked with blue square. Amminoacid residues with 100% similarity are reported in red, in green with 80% similarity and with 60% in grey.

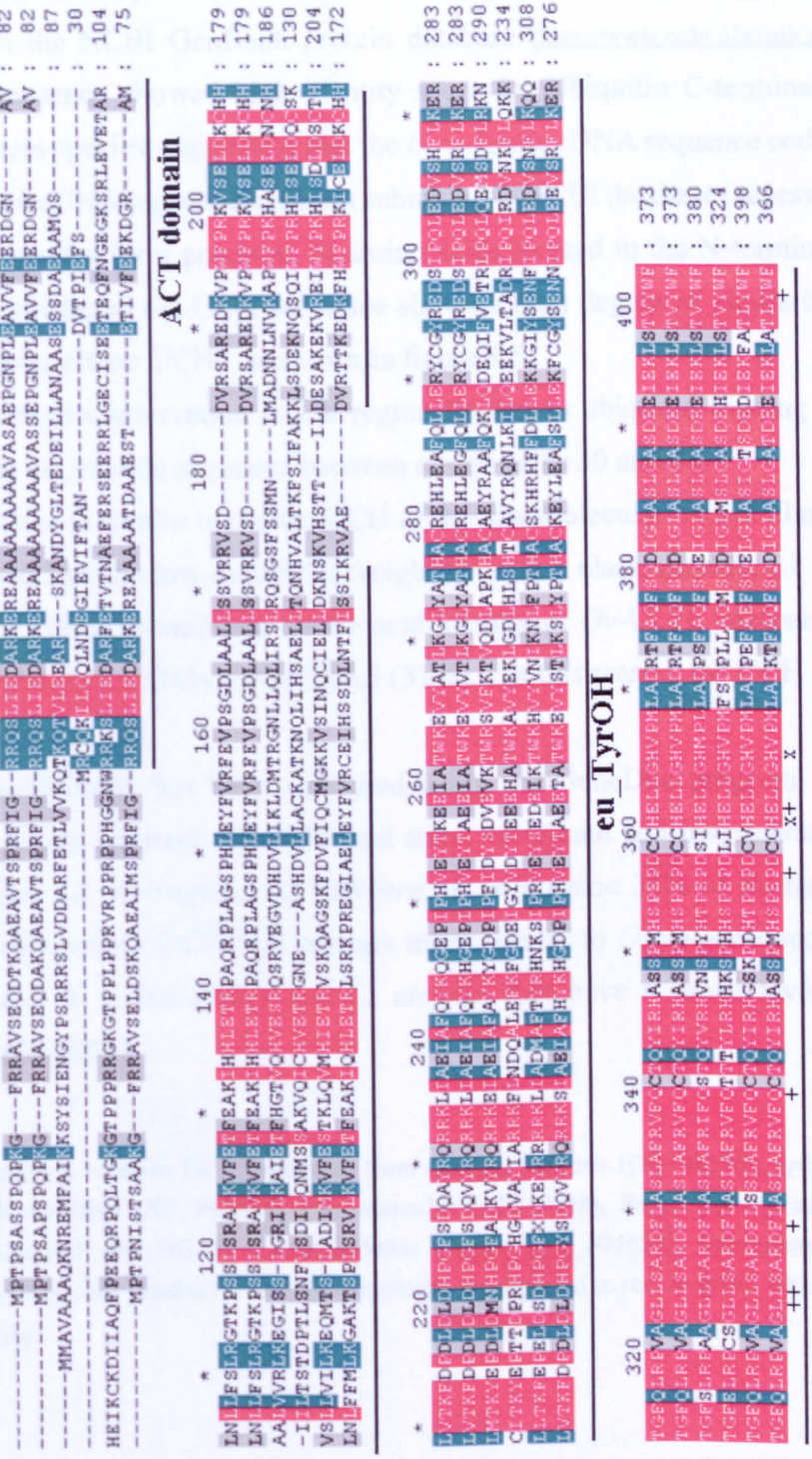

C.quinquefasciatus
B.mori
A.aegypti
D.melanogaster
R.norvegicus
M.musculus
O.vulgaris
45
58
67
76
93
274

4.3.4 Octopus vulgaris Tyrosine hydroxylase

In order to search for the *O. vulgaris* cDNA sequence coding for tyrosine hydroxylase, the most conserved regions of orthologous sequences from vertebrate and invertebrate species were identified. I used degenerate oligonucleotides (table 4.2) to amplify a fragment of 558 bp (Sirakov *et al.*, 2009). Comparing the query sequence (*O. vulgaris* cDNA sequence) with the protein sequences in the database found similarity with orthologous sequences, thus identifying unambiguously the *O. vulgaris* sequence as tyrosine hydroxylase. Subsequently, 1199 bp fragment of *Ov-TH* cDNA was isolated from a *O. vulgaris'* cDNA library using no degenerate primers (table 4.2). I obtained a partial cDNA sequence potentially encoding protein 398 amino acids long. The *Ov-TH* cDNA sequence has been submitted to NCBI database (accession number: FJ617442).

The identified *Ov*-TH sequence was aligned with other known orthologous sequences as reported in figure 4.4. Based on a comparison of the amino acid sequence of *Ov*-TH with vertebrate and invertebrate TH, two structural domains can be distinguished: the C-terminal part is highly conserved and corresponds to the catalytic domain, whereas the N-terminal region involved in the regulation of TH activity is more divergent. This region contains the ACT domain. It is commonly involved in specifically binding an amino acid or other small ligand leading to the regulation of the enzyme. The ACT domain has been detected in a number of diverse proteins; some of these proteins are involved in amino acid and catecholamine biosynthesis. The C-terminal region is specific to eukaryotic tyrosine hydroxylase proteins and contains the amino acid residues (Leu^{319}, Leu^{320}, Phe^{325}, Gln^{335}, Pro^{352}, Glu^{357}, Tyr^{396}) responsible for the binding with the tetrahydrobiopterin (BH4) cofactor. This molecule, together with oxygen, is essential for the hydroxylation of tyrosine to form DOPA. The enzyme uses tyrosine, molecular oxygen, and BH4 to generate 3,4-dihydroxyphenylalanine (DOPA), dihydrobiopterin (BH2) and H_2O (reviewed in Fitzpatrick, 1999). In addition to the listed substrates, TH requires ferrous iron for its reaction (reviewed in Kumer and Vrana, 1996) and the C-terminal region contains some metal binding amino acidic residues His^{356}, His^{361}. From the overall analysis of the *Ov*-TH sequence, it appears to be more closely related to vertebrate (*M. musculus* 44%, *R. norvegicus* 45%) TH than to *Drosophila* and *Caenorhabditis* TH (39% and 31% of similarity, respectively). In particular, the *Ov*-TH shows higher similarity with vertebrates in the N-terminal region, where in general the identity score of sequences between species is very low. In spite of the evolutionary divergence between octopus and vertebrates, these results demonstrate that the gene similarity between them should be remarkable.

Figure 4.4 –Comparison between TH sequences from *Mus musculus* (NP_033403), *Rattus norvegicus* (NP_036872) *Drosophila melanogaster* (CAA53802), *Caenorhaditis elegans* (P90986), *Octopus vulgaris* (FJ617442), *Taeniopygia guttata* (XP_002198967). Black lines are used to highlight two characteristic domains: ACT regulatory domain and eukaryotic tyrosine hydroxylase domain (eu_TyrOH). Amminoacid residues with 100% identity are reported in red, with 80% identity in green and with 60% in grey. The amminoacid residues marked with (+) are known to be involved into the binding of a cofactor and with (x) are indicated metal binding residues.



4.3.5 Octopus vulgaris ubiquitin C-terminal hydrolase

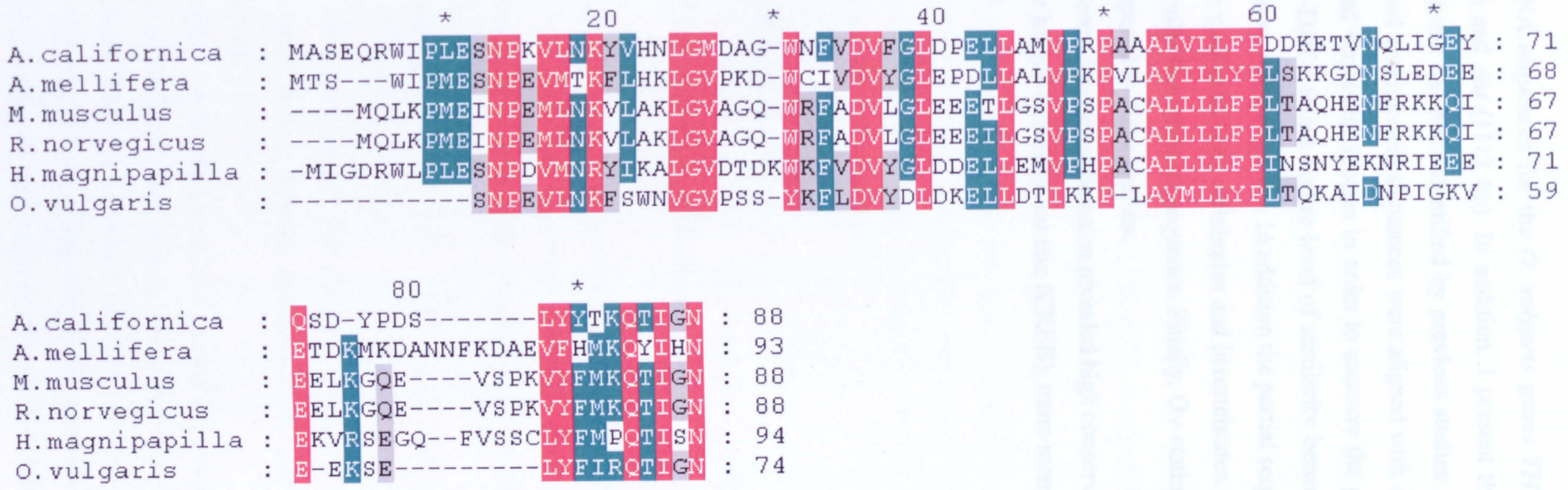
In order to identify the ubiquitin hydrolase cDNA sequence in *Octopus vulgaris*, I analyzed the alignments of orthologous sequences of vertebrate and invertebrate species. Subsequently, I designed degenerate and non-degenerate oligonucleotides (table 4.2) to amplify a fragment 224 bp long. The identity of PCR amplicon was revealed by blasting (blastx) cDNA sequence against the NCBI GenBank protein database (http://www.ncbi.nlm.nih.gov/BLAST/). The identified sequence showed high identity score for Ubiquitin C-terminal Hydrolase proteins of different species, suggesting that the *O. vulgaris* cDNA sequence codes for UCH. The partial *Ov-uch* cDNA sequence has been submitted to NCBI database (accession number: GQ148556). It encodes for a potential 74 amino acids located in the N-terminal region of the protein. The predicted *Ov*-UCH sequence shows a high degree of similarity to known vertebrate and invertebrate UCHs as shown in figure 4.5.

Ov-UCH shows high conservation in the region known as ubiquitin binding site 1. This region comprises the peptide sequence between amino acids 30 and 46.

O.vulgaris sequence is similar to mouse UCH of the low molecular weight class. There are two major members of the low molecular weight hydrolase class, namely, L1 and L3 (e.g. Wilkinson *et al.*, 1995). Overall, the amino acid identity of *Ov*-UCH compared to mouse sequences is higher for L1 (34%), than for L3 (31%). For this reason, the UCH L1 sequence is reported in 4.5.

Overall sequence identity has been measured using the GeneDoc program. Amino acid homology is the same between *Ov*-UCH and some vertebrate and invertebrate sequences (e.g.: *M. musculus* , *R. norvegicus, A. mellifera, A. californica* 34% of similarity), except for *Hydra magnipapollata* UCH that appears more similar to *O. vulgaris* sequence (36% of similarity) and *D. melanogaster* and *C. elegans* that have a lower level of identity (respectivelly 16%, 28%).

Figure 4.5 –Comparison between UCH sequences from *Octopus vulgaris* (GQ148556), *Aplysia californica* (AAB52410), *Apis mellifera* (XP_392902), *Mus musculus* (AF247358), *Rattus norvegicus* (BAA01541), *Hydra magnipapilla* (XP_002156343), *Caenorhabditis elegans* (NP_504654), *Drosophila melanogaster* (NP_524003). Amminoacidic residues with 100% similarity are reported in red, in green with 80% similarity and with 60% in grey.


A.californica : MASEQRWIPLESNPKVLNKYVHNLGMDAG-WNFVDVFGLDPELLAMVPRPAAALVLLFPDDKETVNQLIGEY : 71
A.mellifera : MTS---WIPMESNPEVMTKFLHKLGVPKD-WCIVDVYGLEPDLLALVPKPVLAVILLYPLSKKGDNSLEDEE : 68
M.musculus : ----MQLKPMEINPEMLNKVLAKLGVAGQ-WRFADVLGLEEETLGSVPSPACALLLLFPLTAQHENFRKKQI : 67
R.norvegicus : ----MQLKPMEINPEMLNKVLAKLGVAGQ-WRFADVLGLEEEILGSVPSPACALLLLFPLTAQHENFRKKQI : 67
H.magnipapilla : -MIGDRWLPLESNPDVMNRYIKALGVDTDKWKFVDVYGLDDELLEMVPHPACAILLLFPINSNYEKNRIEEE : 71
O.vulgaris : ----------SNPEVLNKFSWNVGVPSS-YKFLDVYDLDKELLDTIKKP-LAVMLLYPLTQKAIDNPIGKV : 59
A.californica : QSD-YPDS-------LYYTKQTIGN : 88
A.mellifera : ETDKMKDANNFKDAEVFHMKQYIHN : 93
M.musculus : EELKGQE----VSPKVYFMKQTIGN : 88
R.norvegicus : EELKGQE----VSPKVYFMKQTIGN : 88
H.magnipapilla : EKVRSEGQ--FVSSCLYFMPQTISN : 94
O.vulgaris : E-EKSE---------LYFIRQTIGN : 74

4.4 Conclusions

I identified partial cDNA sequences for the *O. vulgaris* genes *TH* (1199 bp), *uch* (224 bp), *Stathmin* (871 bp) and *dat* (1113 bp). In addition, I present the cDNA sequence of *O. vulgaris creb* (4313) that has been identified by previous studies conducted in Fiorito's laboratory. The predicted amminoacid sequences were aligned with orthologous sequences of other invertebrate and vertebrate species in order to measure the percentage of identity. For Ov-CREB and Ov-DAT I found higher level of similarity between octopus and other invertebrates rather than vertebrate species. In addition the partial sequence about 200 bp of Ov-UCH resulted to be similar to both vertebrates and invertebrates. On the other hand, *O. vulgaris* TH is more similar to vertebrate sequence. Finally, Ov-stathmin has very low level of similarity when compared to other species.

The comparison of different functional domains revealed high conservation across species as occurred in my case for kinase-inducible domain (CREB), trans-membrane domains (DAT) and ACT domain (TH).

CHAPTER 5

SPATIAL EXPRESSION OF GENES OF INTEREST IN *O. VULGARIS* CENTRAL NERVOUS SYSTEM

5.1 *In situ* hybridization experiments to study the spatial expression of target genes in the *O. vulgaris* CNS

To document the presence *of alpha tubulin, Ov-creb, Ov-stm, Ov-ubi, Ov-uch, Ov-dat and Ov-TH* in the brain of *O. vulgaris*, I carried out *in-situ* hybridization experiments by using both single or double *in situ* hybridization analysis in order to examine the spatial expression of each gene of interest and, in some cases, to study the possible co-localization of two different messenger RNAs in the same territory.

5.2 Materials and methods

5.2.1 Animals and samples preparation

The brains of naïve octopuses (control group, N = 4) have been serially sectioned as reported below (see paragraph 7.2.2). The sections were post-fixed and then utilized for *in situ* hybridization experiments to localize the expression of genes reported in the table 5.1.

5.2.2 Riboprobe synthesis

Fragments of *Ov-creb, Ov-dat, Ov-stm, Ov-TH, Ov-tubA, Ov-ubi* and *Ov-uch*, were amplified from cDNA templates by PCR with program described in the paragraph 4.2.1 and using specific primers (Table 5.1).

PCR products were analyzed by electrophoresis and purified from agarose gel (see paragraph 4.2.2) and cloned into pCRIITOPO (Invitrogen; see paragraph 4.2.3) according to the manufacturer's instructions. The plasmids were used to transform bacterial cells and the positive clones were selected by PCR screening (for experimental procedures see paragraphs 4.2.4 and 4.2.5). The positive clones were used for plasmid DNA extraction following the

protocol reported in the paragraph 4.2.7. The identity of inserts confirmed by sequencing. Both antisense- and sense-digoxigenin- or fluorescein-labelled RNA probes were generated using a Dig-RNA labelling kit or Flu-RNA labelling kit (Roche, Indianapolis, IN), following the manufacturer's instructions using 1 µg of linearized plasmid (for plasmid linearization see paragraph below, 5.2.2.1). Synthesis reaction were conducted at 37 °C for 2 hours; the DNase I (RNase free) was added (1U/µl) to remove the DNA template. The mix was incubated for another 20 minutes at 37 °C and the reaction was stopped by adding EDTA pH 8 to a final concentration of 25 mM. To remove the unincorporated nucleotides the RNA probes were purified using the Mini Quick Spin RNA Columns G-50 Sephadex (Roche), following manufacturer's instructions. Aliquots of the probe were made and stored at -80 °C.

Table 5.1 : Primers sequences, amplicons size cloned into pCRIITOPO vector and used for riboprobe synthesis

Gene	Primer	Primer sequence 5' - 3'	Amplicon size (bp)
Ov – creb	F	CAGATGGAAGGTTTGCCATT	569
	R	TTCGAAGAGTGAGACCAGCA	
Ov – dat	F	GCCCTAGACGGCATCAAATA	536
	R	CCTCCCTGAGCACAACTAGC	
Ov – stm	F	TGTGGCAGGTATTGTTGGAG	396
	R	TGTTTCCAATCTCTGGGTTTCT	
Ov – th	F	ATTCGAAACGGTTACCAACG	525
	R	AATGCCATGTCTGCAATGAG	
Ov – tuba	F	GCGACCCAGCTACACTAACC	552
	R	AAAGCACGCTTGGCATACAT	
Ov - ub/27A	F	TGTCAAGGCAAAGATTCAAGA	324
	R	GGCCATAAACACACCAGCTC	
Ov – uch	F	TCAAACCCCGAGGTTCTAAAT	224
	R	GCATTACCGATTGTTTGACG	

5.2.2.1 DNA digestion with restriction endonuclease

Plasmid DNA was digested with the suitable restriction endonuclease in a mixture containing 5 units enzyme/1 µg DNA, in a final volume which was at least 20 times more than the enzyme volume, in the presence of 1/10 of a suitable buffer, and at specific temperature suggested by manufacturer's instructions.

5.2.2.2 Riboprobe quantification

For each newly made Dig- or Flu-labelled riboprobe, the concentration was evaluated by dot-blot immunostaining with anti-Dig or anti-Flu antibody AP conjugated (Roche) against a known standard, a labelled RNA control (Roche). RNA dilutions were prepared using the dilution buffer [DEPC H_2O: 20X SSC: formaldehyde (5:3:2)]. Typically, dilutions of the

riboprobes were blotted on Hybond N membrane (Amersham, Little Chalfont, Bucks) with serial dilutions of standard labelled RNA (Roche). The riboprobes were UV-crosslinked to the membrane with Stratalinker for 30 seconds. The filter was first incubated for 30 minutes in blocking solution (5% BSA in 0.1 M maleic acid pH 7.5) and then incubated, 1 hour at rt, in the same solution containing the anti-Dig or anti-Flu alkaline phosphase (AP) antibody (0.15 U/ml). To remove unbound antibodies, two washes in a solution containing 0.1 M maleic acid pH 7.5 and 0.15 M NaCl were done. The filter was equilibrated in the detection solution (100 mM Tris-HCl pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$) and then incubated in the dark in the same solution in which 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (50 mg/ml) and nitroblue tetrazolium (NBT) (50 mg/ml) were added. The AP enzyme produces an insoluble blue precipitate in the presence of these two enzymatic substrates. The coloured compound starts to precipitate in few minutes. The reaction was blocked after 10 minutes by washing the filter with H_2O. The concentrations of experimental riboprobes were estimated by comparing spot intensities of the standard control to the spots of the experimental dilutions.

5.2.3 Double in situ hybridization

Brain sections were pre-warmed at room temperature for 1 hour. Then they were fixed treating with fixation buffer (4% PFA, 0.1 M PBS treated with DEPC) for 1 hour. The sections were rinsed two times in 0.1 M PBS 5 minutes. They were placed in acetylation solution (0.25% acetic anhydride, 1.5% triethanolamine, 0.4% HCl) for 10 minutes. The slides were washed in 2X SSC (treated with DEPC) for 5 minutes, dehydrated with serial dilutions of ethanol. Subsequently, they were treated for 5 minutes with chloroform and rinsed again in 100% and 95% ethanol, placed in the hybridization buffer containing 50% formamide, 5X SSC, 5X Denhardts solution, 100µg/ml Salmon Sperm, 100 µg/ml tRNA, 0.1 ng/µl Dig- and Flu-labelled antisense probes. For control experiments, the brain sections were hybridized with Dig- and Flu-labelled sense-strand probes. The sections were covered with cover slides and incubated overnight in a humidity chamber at 55°C. During post-hybridization stringency washes, the sections were washed in 2X SSC to remove the coverslips and incubated in 2X SSC/ 45% formamide at 50°C for 1 hour. Then the sections were rinsed in NTE for 10 minutes at 37°C and treated with 12.5 µg/ml of RNase A. After these treatments the sections were washed twice in NTE buffer and incubated in 2X SSC for 10 minutes at 45°C and in 2X SSC/ 45% formamide for 30 minutes at 50°C. Then they were washed in PBS and incubated in a quenching solution (1.5% H_2O_2, 0.1 M PBS) for 20 minutes to quench the endogenous peroxidase. The reaction was stopped washing the slides two times in 1X PBS. The sections were equilibrated in TNT buffer (0.1 M Tris pH 7.5, 0.15 M NaCl,

0.05% Tween 20) and incubated for 1 hour at room temperature in blocking buffer TNB (0.5% blocking reagent, 0.1 M Tris pH 7.5, 0.15 M NaCl). Flu epitope detection was done incubating the slices in the TNT buffer containing anti-Flu-HRP (1:500, Roche Diagnostics, USA) overnight in humidity chamber at 4°C. To remove antibody excess, the sections were washed three times for 5 minutes with TNT buffer at room temperature. The sections were equilibrated in 1X TSA Amplification Diluent (PerkinElmer, MA, USA) for 10 minutes and the detection of the antibody was carried out in Flurophore Tyramide Amplification Reagent Cy5 (1:100, PerkinElmer) for 20 minutes at room temperature. The reaction was stopped by washing in TNT buffer. Then the slides were incubated at room temperature for 20 minutes in 1.5% H_2O_2. The quenching reaction was stopped by washing in TNT buffer. The sections were incubated in blocking buffer TNB for 1 hour at room temperature then the primary antibody anti-Dig-HRP (1:500, Roche) was added and the slides were incubated overnight in a humidity chamber at 4°C. To remove unbound antibody the sections were washed in TNT buffer. The detection of bound antibody was done by incubating in Fluorophore Tyramide Amplification Reagent Cy3 (1:100, PerkinElmer) for 20 minutes at room temperature. Then the slices were rinsed in TNT buffer. In order to visualize the cell nuclei, the slices were counterstained with SYTO 13 (Invitrogen). Then the slices were dehydrated using serial dilutions of ethanol and air-dried. The brain sections were examined first using a GenePix Personal 4100A micro-array scanner (Molecular Devices) followed by analysis with a Zeiss confocal laser scanning microscope (LSM 510).

5.3 Results

In the following pages, I have analyzed the expression of two reference genes (alpha-tubulin and ubiquitin) and four target genes (ubiquitin hydrolase, stathmin, dopamine transporter, Tyrosine Hydroxylase) within the different masses of *O. vulgaris* central nervous system. For sake of clarity, the results of expression of each gene are discussed separately.

Plate I-Localization of *alpha-tubulin* mRNA in the octopus brain

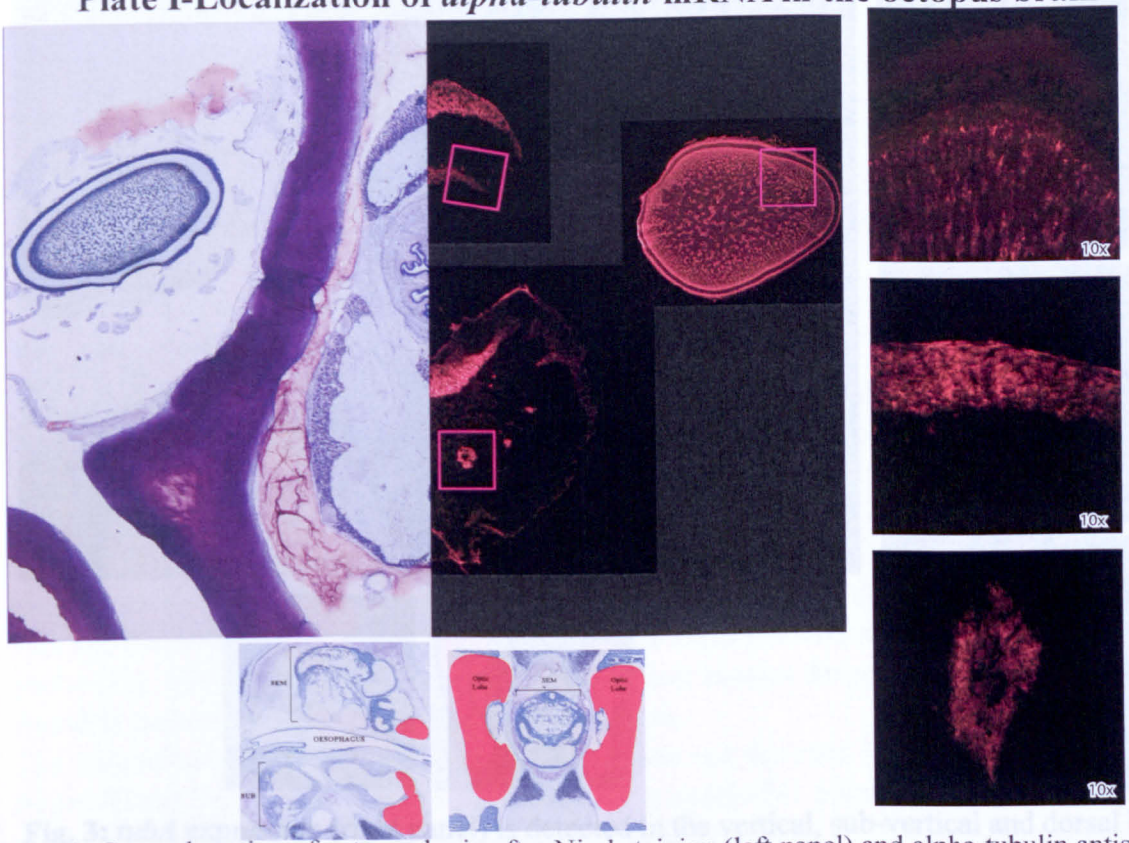


Fig. 1: Coronal section of octopus brain after Nissl staining (left panel) and alpha-tubulin antisense digoxigenin-labelled riboprobe hybridization (right panel). *tubA* expression (right panel) is detected in the superior buccal and inferior frontal lobe (SEM), in the posterior brachial lobe (SUB), and in the medulla, inner and outer layer (OL); for anatomical reference in the left panel see Appendix 3-plate 4. Details (pink square) are presented on the right for each mass (OL, top; SEM, middle; SUB, bottom) with their relative magnifications.

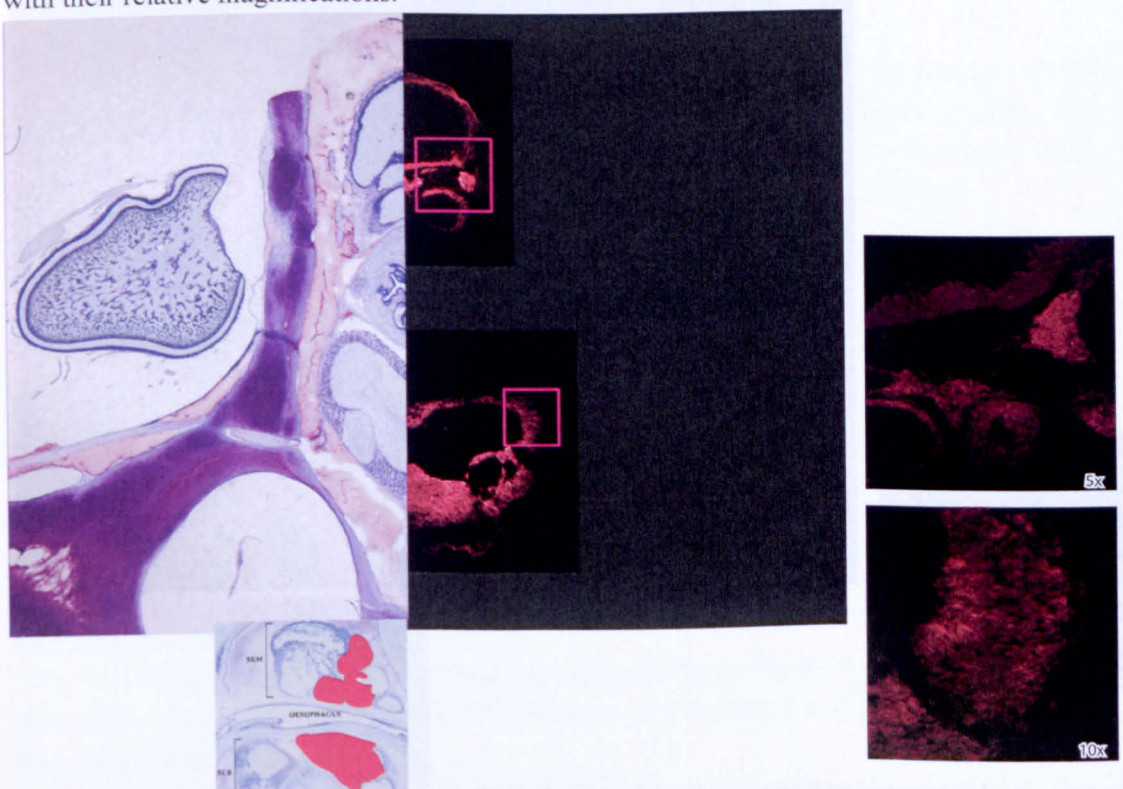


Fig. 2: *tubA* expression (right panel) is detected in the superior frontal lobe, superior lateral frontal lobe, sub-frontal lobe and posterior basal lobe (SEM), in the anterior pedal lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 8. For other details see Fig.1.

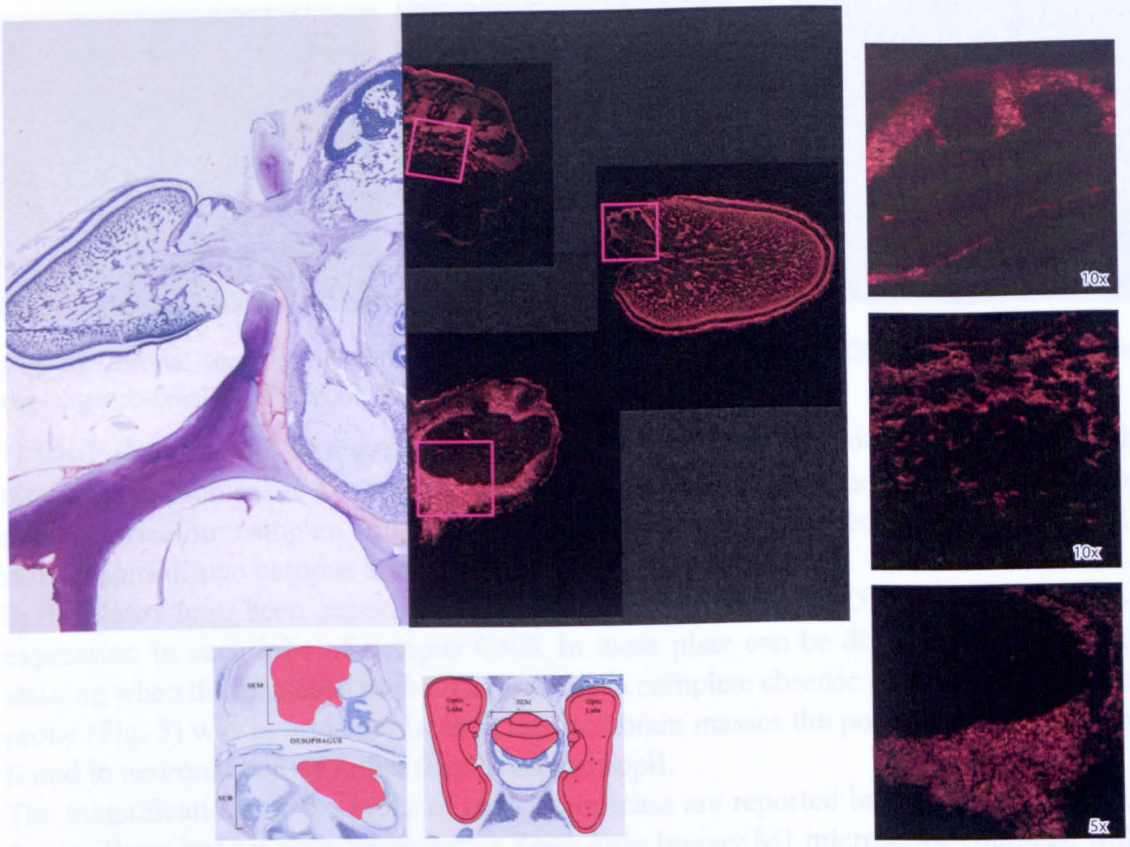


Fig. 3: *tubA* expression (right panel) is detected in the vertical, sub-vertical and dorsal basal lobes (SEM), in the magnocellular and posterior pedal lobe (SUB) and in the medulla, inner and outer layer, optic gland and olfactory lobe (OL). For anatomical reference in the left panel see Appendix 3-plate 12. For other details see Fig.1.

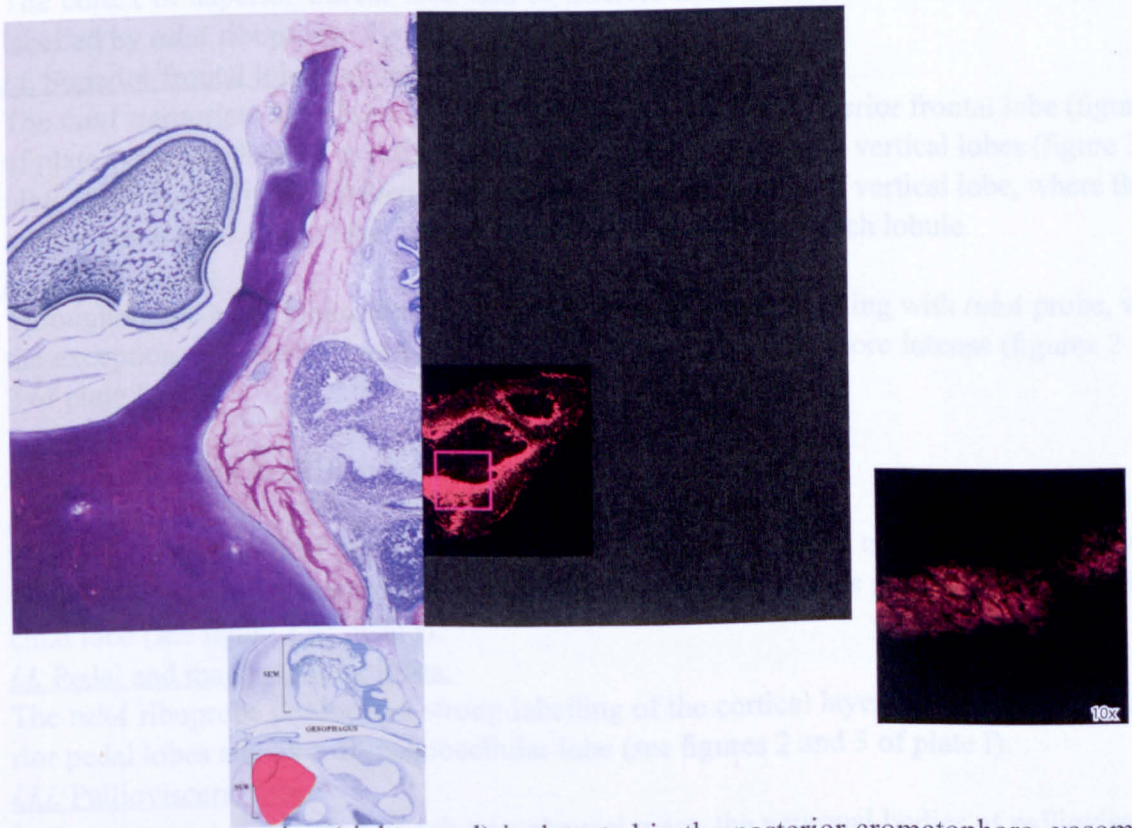


Fig. 4: *tubA* expression (right panel) is detected in the posterior cromatophore, vasomotor and palliovisceral lobes (SUB).). For anatomical reference in the left panel see Appendix 3-plate 14. For other details see Fig.1.

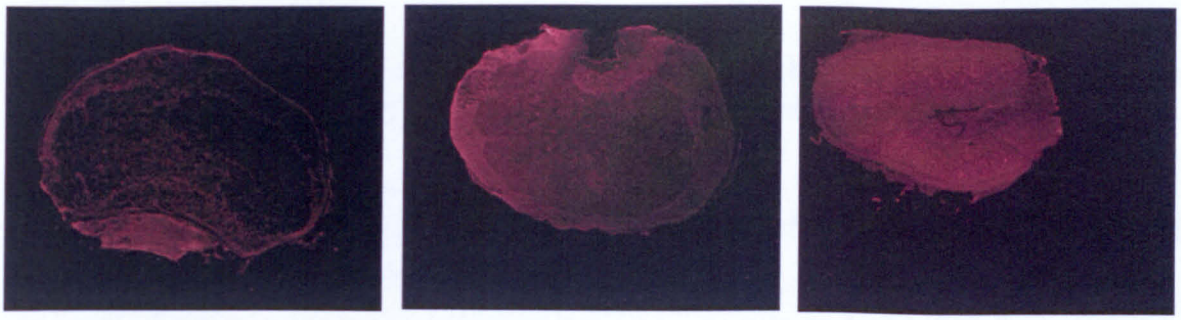


Fig. 5: Coronal sections of octopus brain (SEM, left; SUB, middle; OL, right) after *tubA* sense digoxigenin-labelled riboprobe hybridization.

[1] I used *alpha-tubulin* as a control gene for the *in situ* hybridization experiments. Its ubiquitous expression in the *O. vulgaris* CNS has been confirmed by the results of these experiments carried out using an antisense digoxigenin-labelled riboprobe and 20 μm coronal serial sections of two octopus brains.

In the plate I have been reported several images SEM, SUB and OL representing the *α-tub* expression in each lobe of octopus CNS. In these plate can be distinguished the positive staining when the antisense probe was used and a complete absence of signal when the sense probe (Fig. 5) was hybridized. In each lobes of brain masses the positive signals have been found in neuronal bodies rather than in the neuropil.

The magnifications of the lobes of each brain mass are reported in the right panel of each figure. These images were taken with a Zeiss Axio Imager M1 microscope equipped with an Axiocam digital camera.

***tubA* distribution in SEM**

i. Buccal lobe and inferior frontal lobe:

The cortex of superior buccal lobe and of inferior frontal and sub-frontal lobes appeared labelled by *tubA* riboprobe (figures 1 and 2 of plate I).

i.i. Superior frontal lobe and vertical lobe:

The *tubA* transcripts have been found in the cortical region of superior frontal lobe (figure 2 of plate I) and produced a specific labelling of the sub-vertical and vertical lobes (figure 3 of plate I). Cells positively labelled were present inside the lobules of vertical lobe, where there are the larger cells of the lobe, but also in the cortical region of each lobule.

i.i.i. Basal lobes:

Throughout the basal lobes, the brain cortex showed a slight labelling with *tubA* probe, with the exception of posterior basal lobe, whose labelling appeared more intense (figures 2 and 3 of plate I).

***tubA* distribution in SUB**

i. Brachial lobe:

The cells of the cortical layer of brachial lobe expressed *tubA*, no positive signals were found in the neuropil, but *tubA* is expressed in the brachial nerves present in posterior brachial lobe (see figure 1 of plate I).

i.i. Pedal and magnocellular lobes:

The *tubA* riboprobe produced a strong labelling of the cortical layers of anterior and posterior pedal lobes and also of magnocellular lobe (see figures 2 and 3 of plate I).

i.i.i. Palliovisceral lobe:

In the most posterior region of sub-oesophageal mass, the neuronal bodies of palliovisceraland vasomotor lobes appeared strongly labelled by the *tubA* riboprobe (see figure 4 of plate I).

iiii. Chromatophore lobes

In both anterior (see plate IV in Appendix 4) and posterior chromatophore lobes (see figure 4 of plate I), *tubA* mRNAs were found, but they were present only in the cortex.

tubA distribution in OL

tubA gene seemed expressed in both inner and outer layer of the cortex and also in the cell islands of the medulla (see figures 1 and 3 of plate I).

i. Olfactory lobe

Looking at figure 3 of plate I is possible to identify the presence of cells positively labelled in the cortex of olfactory lobe.

PlateII-Localization of *Ov-creb* mRNA in the octopus brain

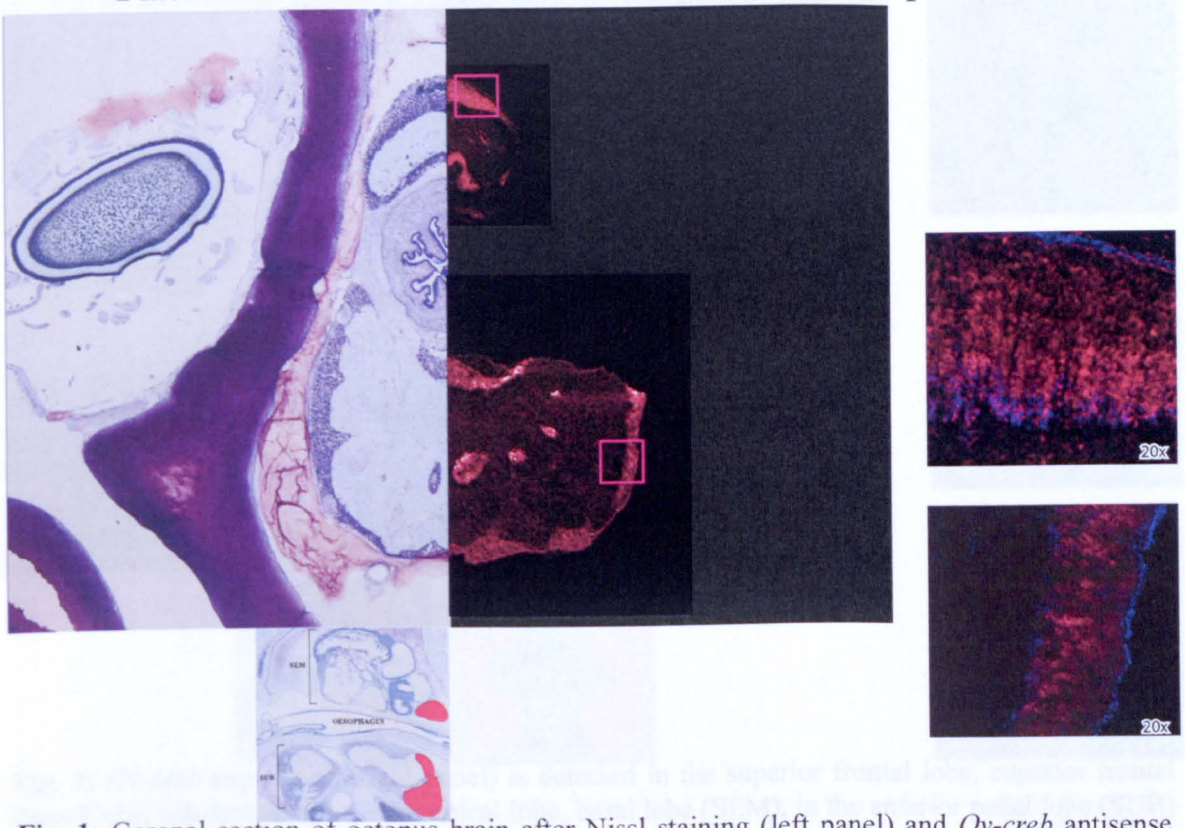


Fig. 1: Coronal section of octopus brain after Nissl staining (left panel) and *Ov-creb* antisense digoxigenin-labelled riboprobe hybridization (right panel).Ov-creb expression (right panel) is detected in the superior buccal and inferior frontal lobes (SEM), in the brachial lobe (SUB); for anatomical reference in the left panel see Appendix 3-plate 4.
Details (pink square) are presented on the right for each mass (SEM, top; SUB, bottom) with their relative magnifications. In the magnification together with the signal of riboprobe is visibile also the cell nuclei with blue staining.

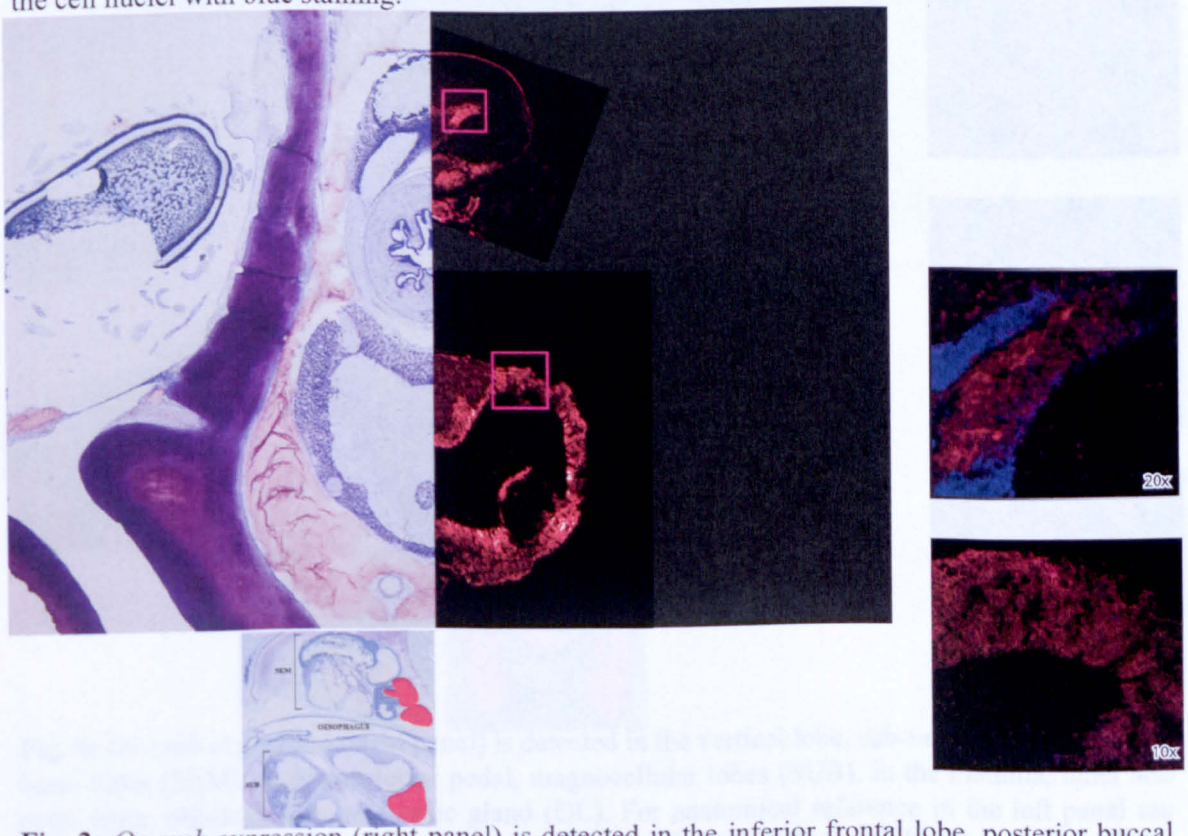


Fig. 2: *Ov-creb* expression (right panel) is detected in the inferior frontal lobe, posterior buccal lobe (SEM) and in the brachial lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 5. For other details see Fig. 1.

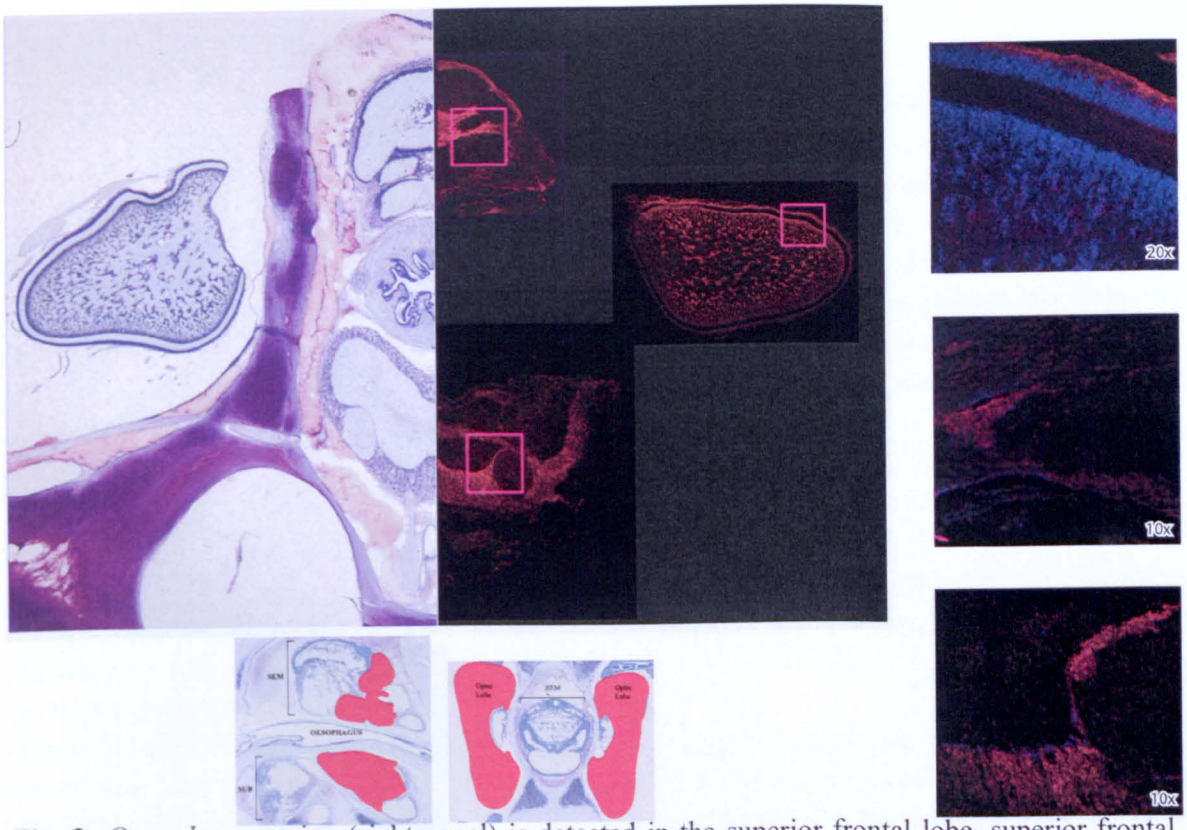


Fig. 3: *Ov-creb* expression (right panel) is detected in the superior frontal lobe, superior frontal lateral lobe, sub-frontal lobe, sub-vertical lobe, basal lobe (SEM), in the anterior pedal lobe (SUB) and in the medulla, inner and outer layer (OL). For anatomical reference in the left panel see Appendix 3-plate 8.
Details (pink square) are presented on the right for each mass (OL, top; SEM, middle; SUB, bottom) with their relative magnification. For other details see Fig. 1.

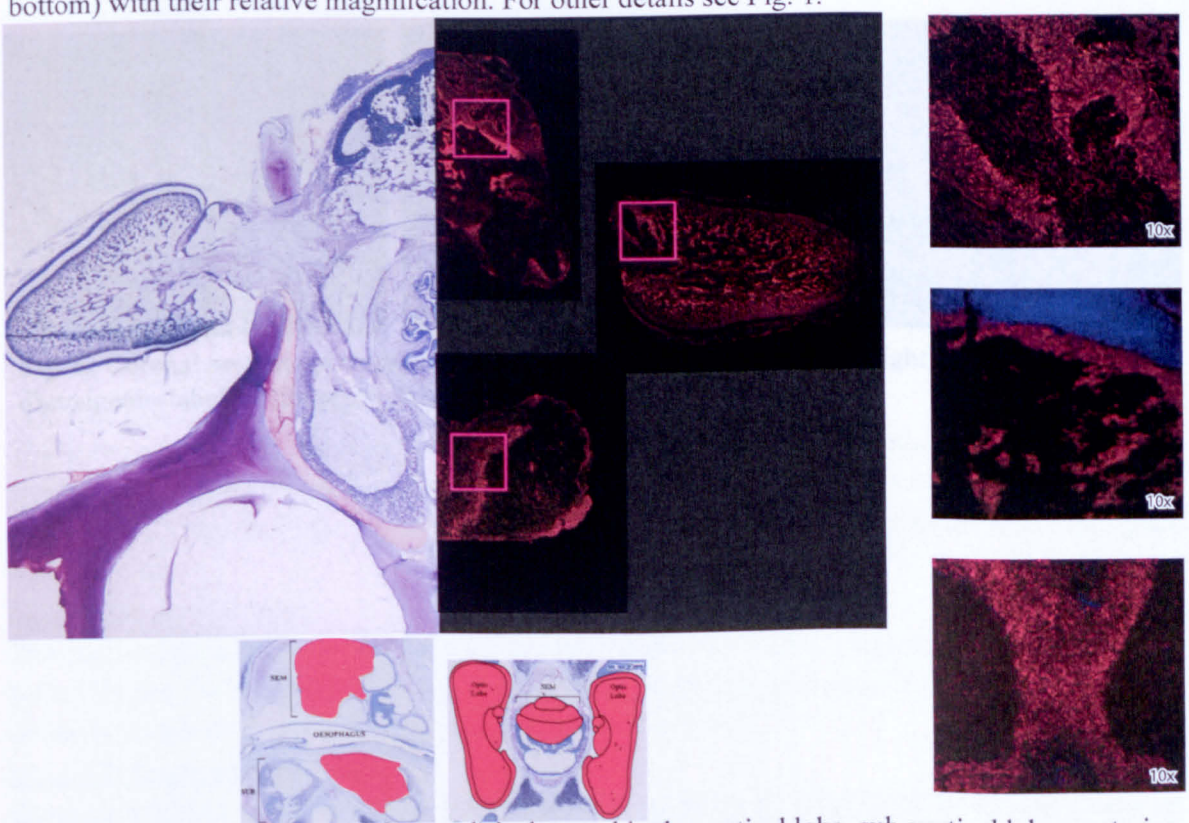


Fig. 4: *Ov-creb* expression (right panel) is detected in the vertical lobe, sub-vertical lobe, posterior basal lobes (SEM), in the posterior pedal, magnocellular lobes (SUB), in the medulla, inner and outer layer, olfactory lobe and optic gland (OL). For anatomical reference in the left panel see Appendix 3-plate 12. Details (pink square) are presented on the right for each mass (OL, top; SEM, middle; SUB, bottom). For other details see Fig. 1.

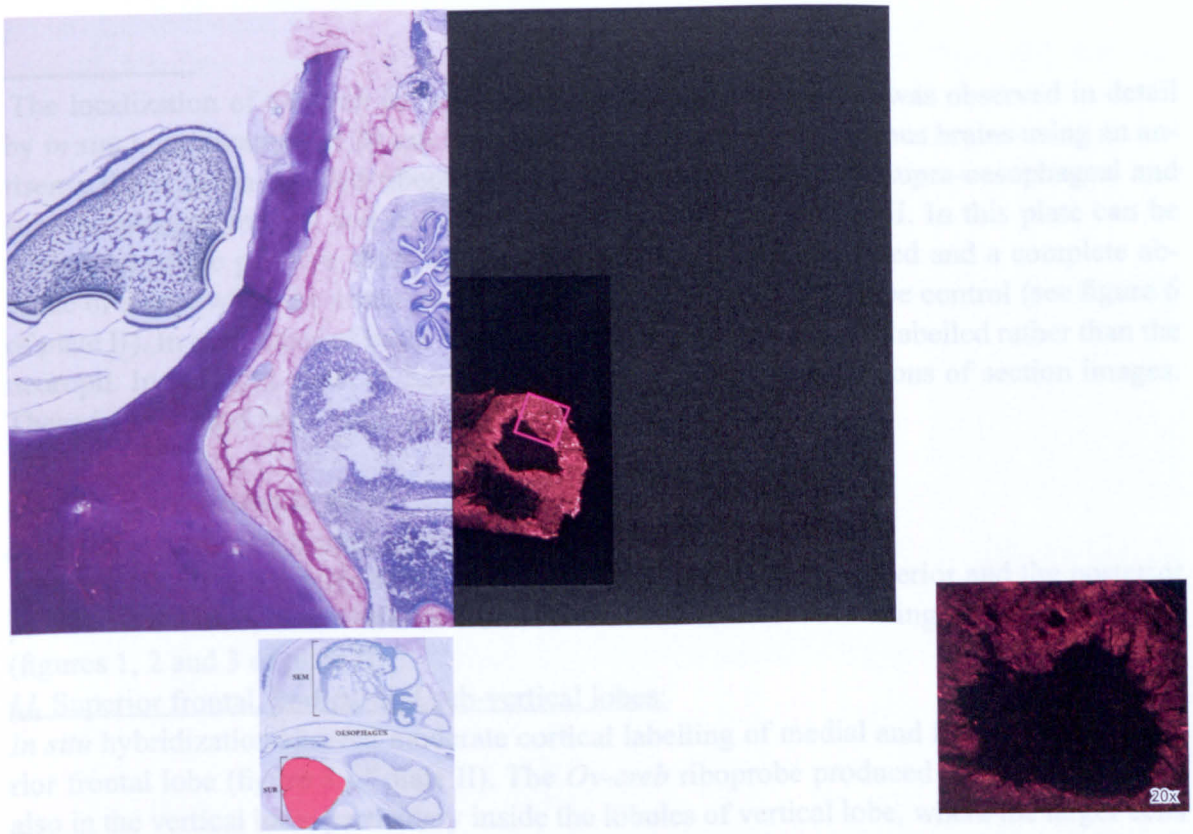


Fig. 5: *Ov-creb* expression (right panel) is detected in the posterior cromatophore lobe and palliovisceral lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 14. For other details see Fig. 1.

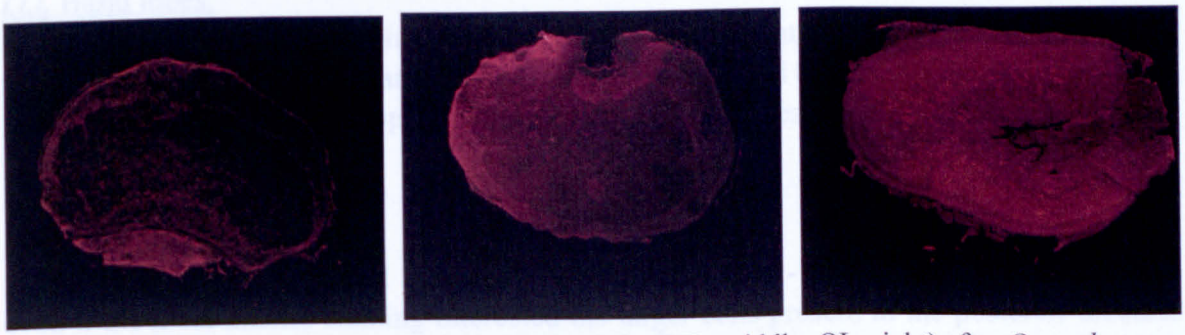


Fig. 6: Coronal sections of octopus brain (SEM, left; SUB, middle; OL, right) after *Ov-creb* sense digoxigenin-labelled riboprobe hybridization.

1

The localization of *Ov-creb* in the octopus central nervous system was observed in detail by *in situ* hybridization on 20 μm coronal serial sections of two octopus brains using an antisense digoxigenin-labelled riboprobe. Several coronal sections of supra-oesophageal and sub-oesophageal masses and optic lobes are presented in the plate II. In this plate can be distinguished the positive staining when the antisense probe was used and a complete absence of signal when the sense probe was used like a specificity probe control (see figure 6 of plate II). In each lobes of brain masses the cortex only was mainly labelled rather than the neuropil. In the right panel of figures are reported some magnifications of section images. These images have been made using the confocal microscope.

Ov-creb distribution in SEM

i. Buccal and inferior frontal lobes:

A strong labelling has been found in the cortical regions of both superior and the posterior part of buccal lobe, in the inferior frontal and in sub-frontal lobes using *Ov-creb* riboprobe (figures 1, 2 and 3 of plate II).

i.i. Superior frontal, vertical and sub-vertical lobes:

In situ hybridization showed moderate cortical labelling of medial and lateral part of superior frontal lobe (figure 3 of plate II). The *Ov-creb* riboprobe produced a specific labelling also in the vertical lobe, particulary inside the lobules of vertical lobe, where the larger cells of the lobe are present (see figure 4 of plate II).

Strongly positive cells were found in the sub-vertical lobe where are present several islands of cells which seemed to contain large quantity of *Ov-creb* mRNA (see figures 3 and 4 of plate II).

i.i.i. Basal lobes:

Throughout the basal lobes, the brain cortex showed a slight labelling *Ov-creb* riboprobe, with the exception of dorsal part of the anterior basal lobe and the dorsal part of the posterior basal lobe, whose labelling appeared more intense (see figures 3 and 4 of plate II).

Ov-creb distribution in SUB

i. Brachial lobes:

In situ hybridization revealed a strong labelling of both pre- and posterior brachial lobes, even if the most intense staining interested the larger cells present in the posterior brachial lobe (see figure 1 and 2 of plate II).

i.i. Pedal and magnocellular lobes:

The cortical layers of anterior (see figures 3 of plate II), middle (see plate V of Appendix 4) and posterior pedal lobes showed strongly labelled cells (see figure 4 of plate II), instead a more slightly staining was found in the cells belonging to the magnocellular lobe (see figure 4 of plate II).

i.i.i. Palliovisceral lobe:

The palliovisceral lobe in the most posterior region of sub-oesophageal mass appeared labelled by the *Ov-creb* riboprobe, demonstrating the strong presence of *Ov-creb* mRNA particularly in the most posterior part (see figure 5 of plate II).

i.i.i.i. Chromatophore lobes:

Ov-creb mRNAs were found in both anterior (see plate IV of Appendix 4) and posterior chromatophore lobes, even if these transcripts seemed more abundant in the posterior ones (see figure 4 of plate II).

***Ov-creb* distribution in OL**

A strong labelling has been found in the optic lobes (see figures 3 and 4 of plate II), the positive signals were present in inner and outer layers of the cortex, but also in the island cells of the medulla. Instead, in the middle part of the optic lobe, the region more closely related to optic tract, the signals seemed present only in the medulla rather than in the cortex (see figure 4 of plate II).

i. Olfactory lobe and optic gland:

The cortical regions of both the olfactory lobe and the optic gland were strongly labelled by the *Ov-creb* riboprobe (see figure 4 of plate II).

Plate III-Localization of *stathmin* mRNA in the octopus brain

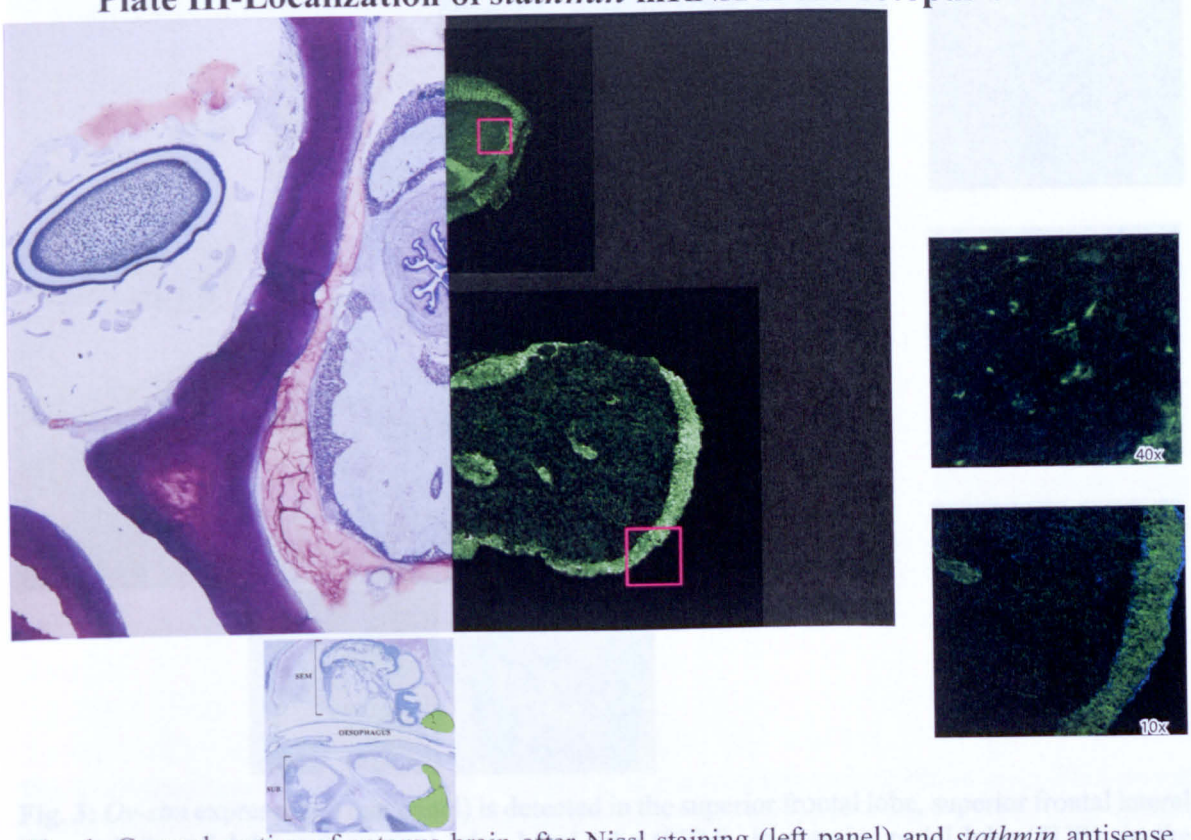


Fig. 1: Coronal section of octopus brain after Nissl staining (left panel) and *stathmin* antisense fluorescein-labelled riboprobe hybridization (right panel). *Ov-stm* expression (right panel) is detected in the superior buccal lobe, inferior frontal lobe (SEM) and in the brachial lobe (SUB); for anatomical reference in the left panel see Appendix 3-plate 4.
Details (pink square) are presented on the right for each mass (SEM, top; SUB, bottom) with their relative magnifications. In the magnification together with the signal of riboprobe is visibile also the cell nuclei with blue staining.

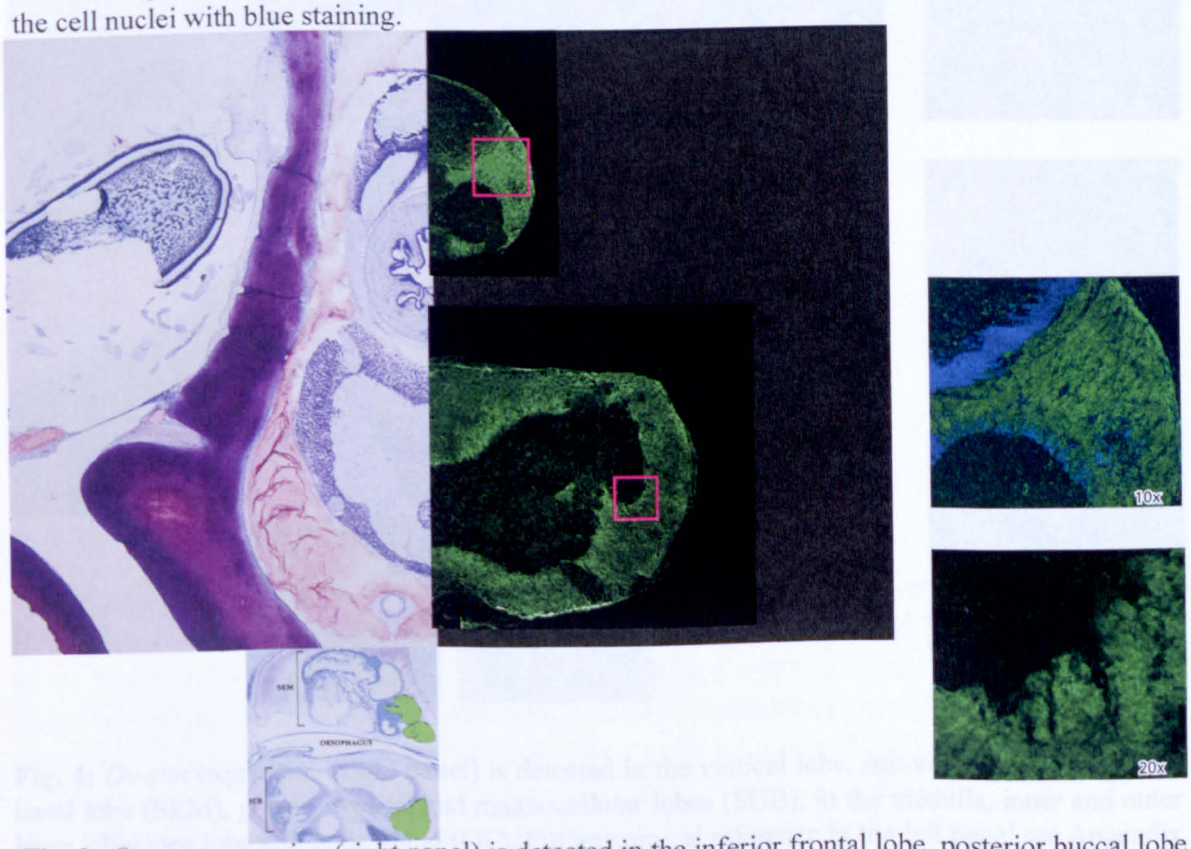


Fig. 2: *Ov-stm* expression (right panel) is detected in the inferior frontal lobe, posterior buccal lobe (SEM) and in the brachial lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 5. For other details see Fig. 1.

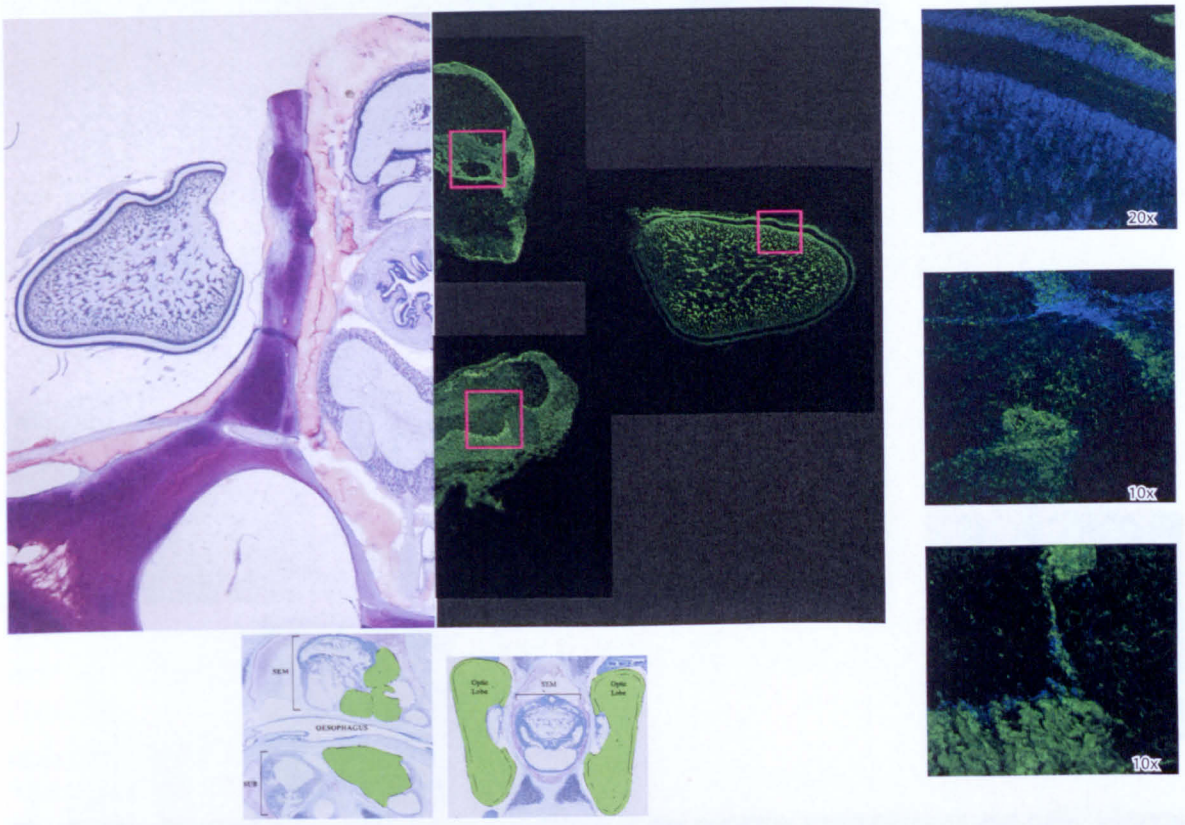


Fig. 3: *Ov-stm* expression (right panel) is detected in the superior frontal lobe, superior frontal lateral lobe, sub-frontal lobe, sub-vertical lobe, basal lobe (SEM), in anterior pedal lobe (SUB), in the medulla, in the inner and outer layer (OL). For anatomical reference in the left panel see Appendix 3-plate 8. For other details see Fig. 1.

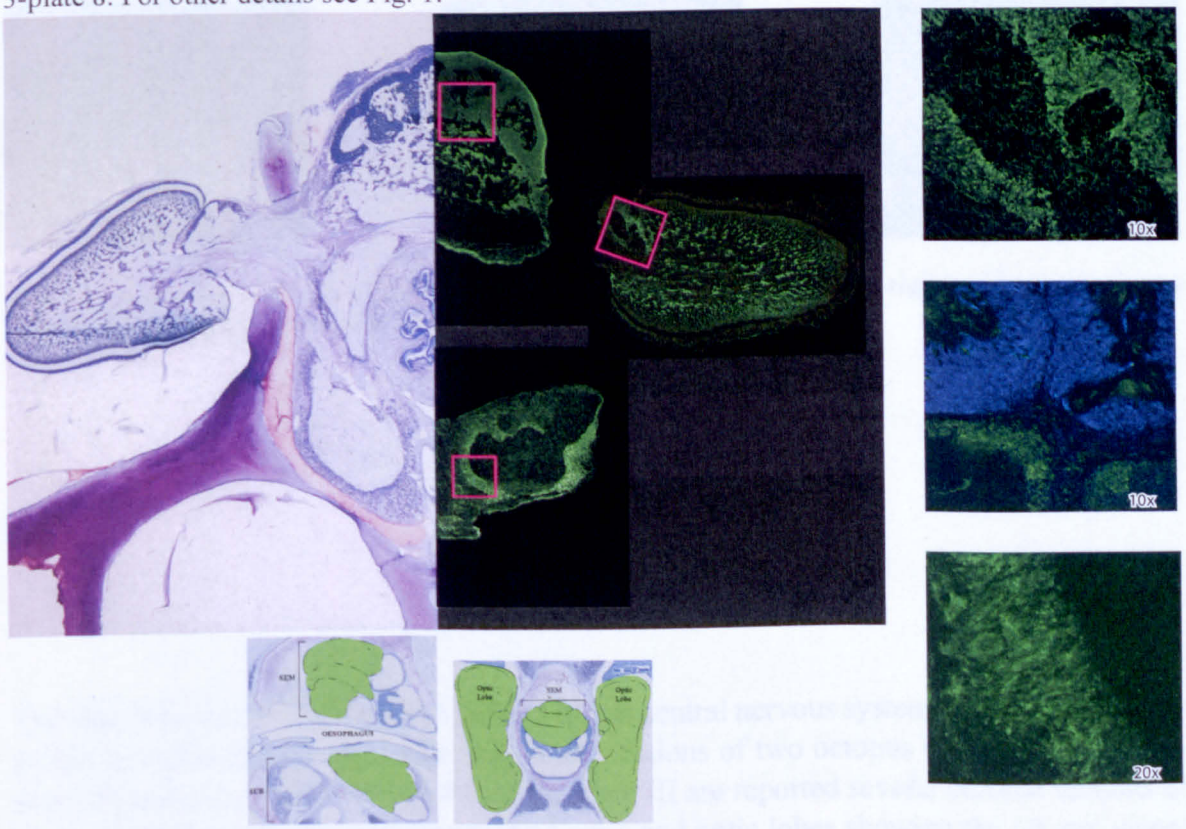


Fig. 4: *Ov-stm* expression (right panel) is detected in the vertical lobe, sub-vertical lobe, posterior basal lobe (SEM), posterior pedal and magnocellular lobes (SUB), in the medulla, inner and outer layer, olfactory lobe and optic gland (OL). For anatomical reference in the left panel see Appendix 3-plate 12. For other details see Fig. 1.

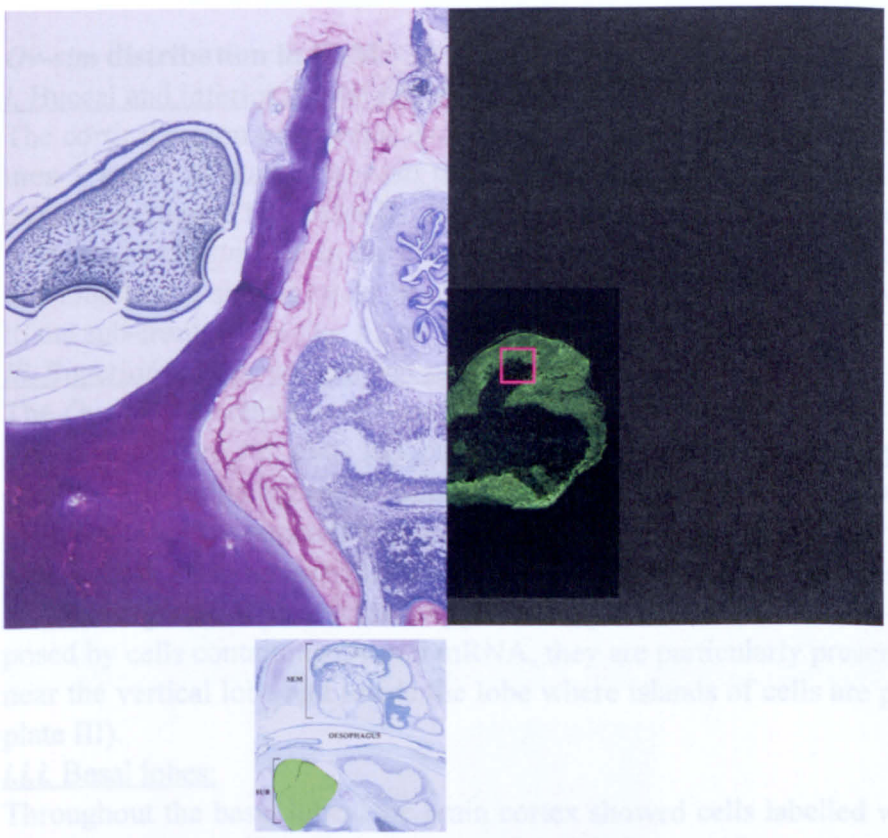


Fig. 5: *Ov-stm* expression (right panel) is detected in the posterior cromatophore and palliovisceral lobes (SUB). For anatomical reference in the left panel see Appendix 3-plate 14. For other details see Fig. 1.

Fig. 6: Coronal sections of octopus brain (SEM, left; SUB, middle; OL, right) after *Ov-stm* sense fluorescein-labelled riboprobe hybridization.

The distribution of *Ov-stm* mRNA in the octopus central nervous system has been studied by *in situ* hybridization on 20 µm coronal serial sections of two octopus brains using an anti-sense fluorescein-labelled riboprobe. In the plate III are reported several coronal sections of supra-oesophageal mass, sub-oesophageal mass and optic lobes showing the *Ov-stm* signal distribution in octopus CNS. In the figure 6 of plate III are included the control of probe bond specificity obtained by sense riboprobe hybridization. In each lobes of brain masses the cortex was mainly labelled, but in some cases also the neuropil showed the presence of *Ov-stm* transcripts. In the right panel of the figures shown in this plate are reported some magnifications which have been made using the confocal microscope.

***Ov-stm* distribution in SEM**

i. Buccal and inferior frontal lobes:

The cortical region of superior and posterior buccal lobes appeared positively labelled (figures 1 and 2 of plate III). Also the neuropil of the superior buccal lobe showed *Ov-stm* transcripts (figure 1 of plateIII). *In situ* hybridization experiments showed a clear signal of presence of *Ov-stm* also in the inferior frontal lobe (see figure 1 and 2 of plate III). Inside of this lobe strong signals could be observed in the lateral region (see Appendix 4-plate I) and in the sub-frontal lobe (see figure 3 plate III).

ii. Superior frontal, vertical and sub-vertical lobes:

The *Ov-stm* transcripts have been found in the superior frontal lobe, securely in its cortical region (see figure 3 of plate III), but in some cases also in the central part of lobe. This is the case showed in the figures of Appendix 4-plate II, where the *Ov-stm* signal was present in the neuropil of the superior frontal lobe.

The vertical lobe showed only few labelled cells, they are present inside the lobules of this lobe (see figure 4 of plate III and Appendix 4- plate III). Also, the sub-vertical lobe was composed by cells containing *Ov-stm* mRNA, they are particularly present in the cortical region near the vertical lobe and inside the lobe where islands of cells are present (see figure 4 of plate III).

iii. Basal lobes:

Throughout the basal lobes, the brain cortex showed cells labelled with *Ov-stm* riboprobe, there are not exceptions for any parts of these lobes (see figures 3 and 4 of plate III; but see also Appendix 4-plateII).

***Ov-stm* distribution in SUB**

i. Brachial lobes:

The cells of the cortical layer of both pre-brachial and posterior brachial lobes appeared to express *Ov-stm* (see figures 1 and 2 of plate III), instead no positive signals were found in the neuropil of these lobes.

ii. Pedal and magnocellular lobes:

In situ hybridization revealed a strong labelling of the cortical layers of anterior (see figure 3 of plate III), middle (see Appendix 4-plateV) and posterior pedal lobes (see figure 4 of plate III). Also, the cells of magnocellular lobe appeared to contain *Ov-stm* mRNA as showed in the figure 4 of plate III.

iii. Palliovisceral lobe:

Throughout the most posterior part of sub-oesophageal mass, the brain cortex of the palliovisceral lobe showed cells which contain *Ov-stm* mRNA (see figure 5 of plate III), whereas any positive signals have been found in the neuropil.

iiii. Chromatophore lobes:

In the cortex of anterior (in Appendix 4-plate V) and posterior chromatophore lobes (see figure 5 of plate III) *Ov-stm* mRNAs were found.

***Ov-stm* distribution in OL**

Ov-stm transcripts were found in the optic lobes. The positive signals were more abundant in the outer layer of the cortex in respect to the inner one (see figures 3 and 4 of plate III), but they were also present in the island cells of the medulla.

i. Olfactory lobe and optic gland:

Looking at images 4 of plate III is possible to identify the presence of cells positively labelled in the cortex of both olfactory lobe and optic gland

Plate IV-Localization of *ubiquitin* mRNA in the octopus brain

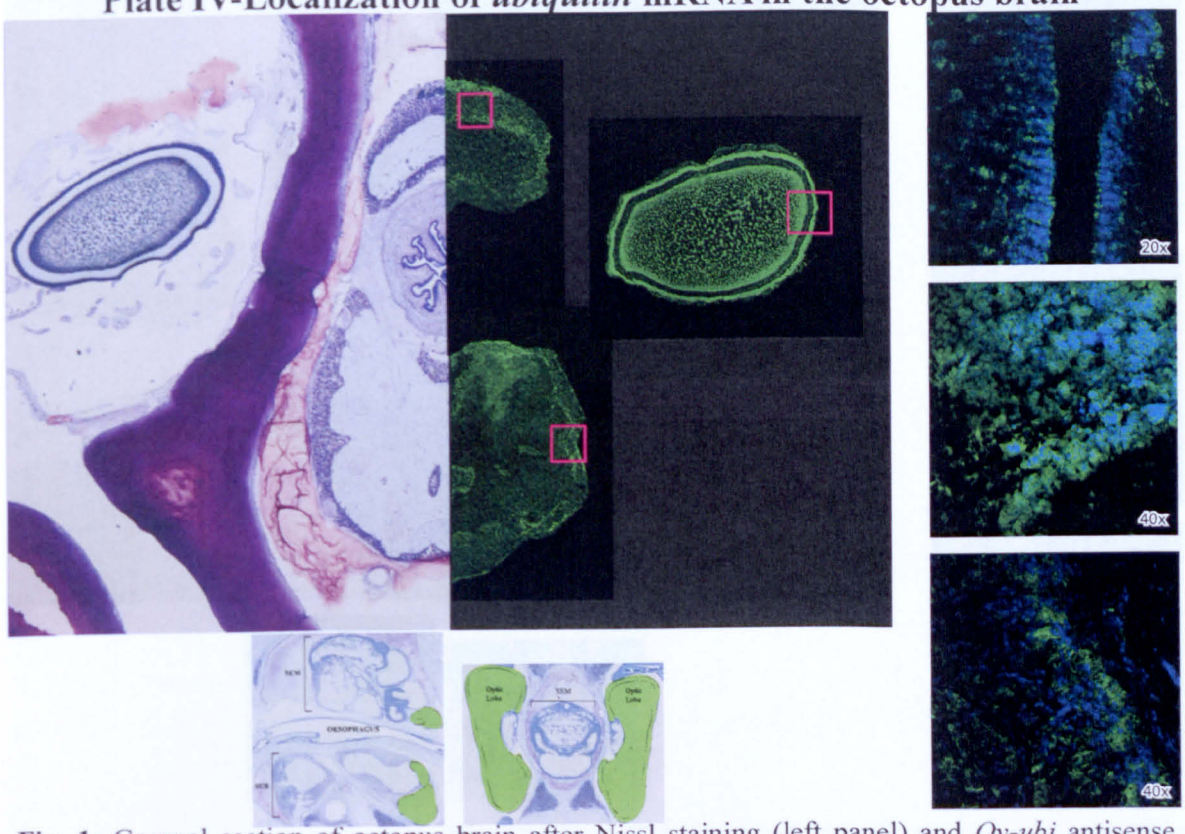


Fig. 1: Coronal section of octopus brain after Nissl staining (left panel) and *Ov-ubi* antisense fluorescein-labelled riboprobe hybridization (right panel). *Ov-ubi* expression (right panel) is detected in the superior buccal lobe, inferior frontal lobes (SEM), in the brachial lobe (SUB), in the medulla, inner and outer layer (OL); for anatomical reference in the left panel see Appendix 3-plate 4. Details (pink square) are presented on the right for each mass (OL, top; SEM, middle; SUB, bottom) with their relative magnifications. In the magnification together with the signal of riboprobe is visibile also the cell nuclei with blue staining.

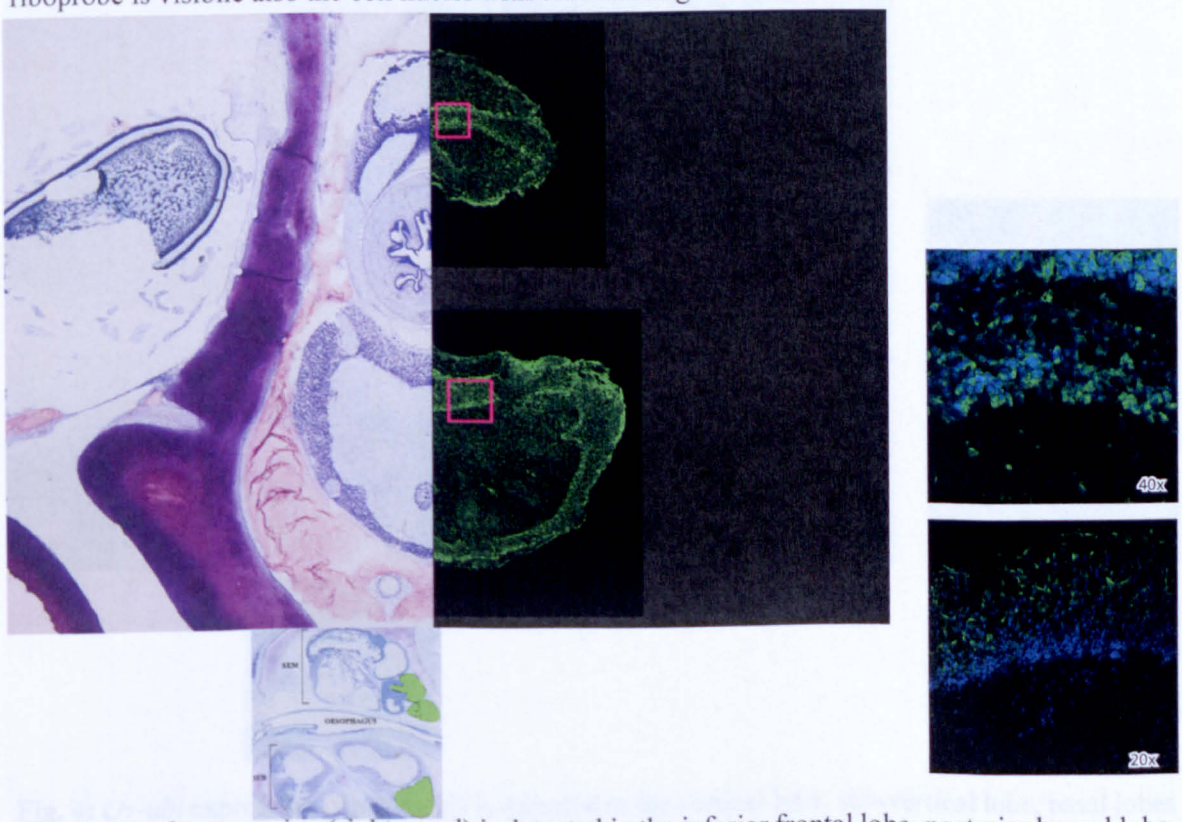


Fig. 2: *Ov-ubi* expression (right panel) is detected in the inferior frontal lobe, posterior buccal lobe (SEM) and in the brachial lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 5. For other details see Fig. 1.

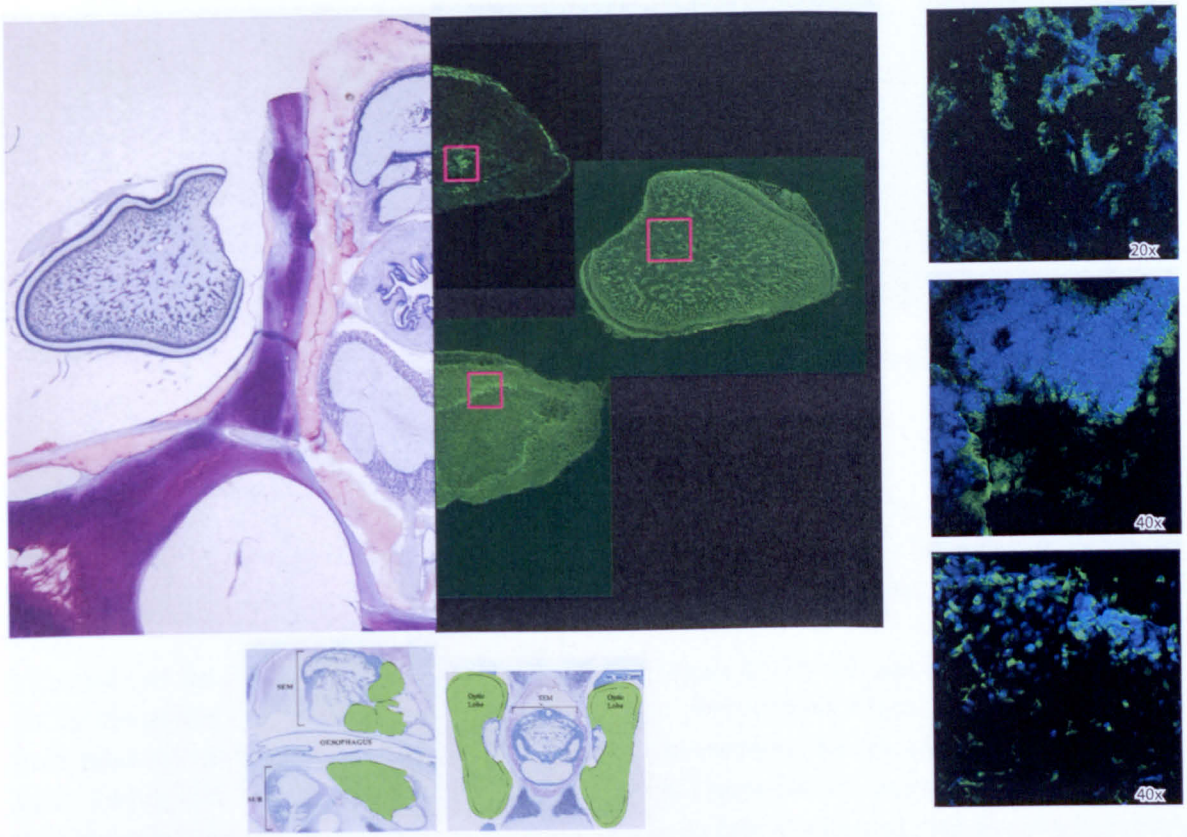


Fig. 3: *Ov-ubi* expression (right panel) is detected in the superior frontal lobe, superior frontal lateral lobe, sub-frontal lobe, inferior frontal lobe, sub-vertical lobe, basal lobe (SEM), in the anterior pedal lobe (SUB) and in the medulla, inner and outer layer (OL). For anatomical reference in the left panel see Appendix 3-plate 8. For other details see Fig. 1.

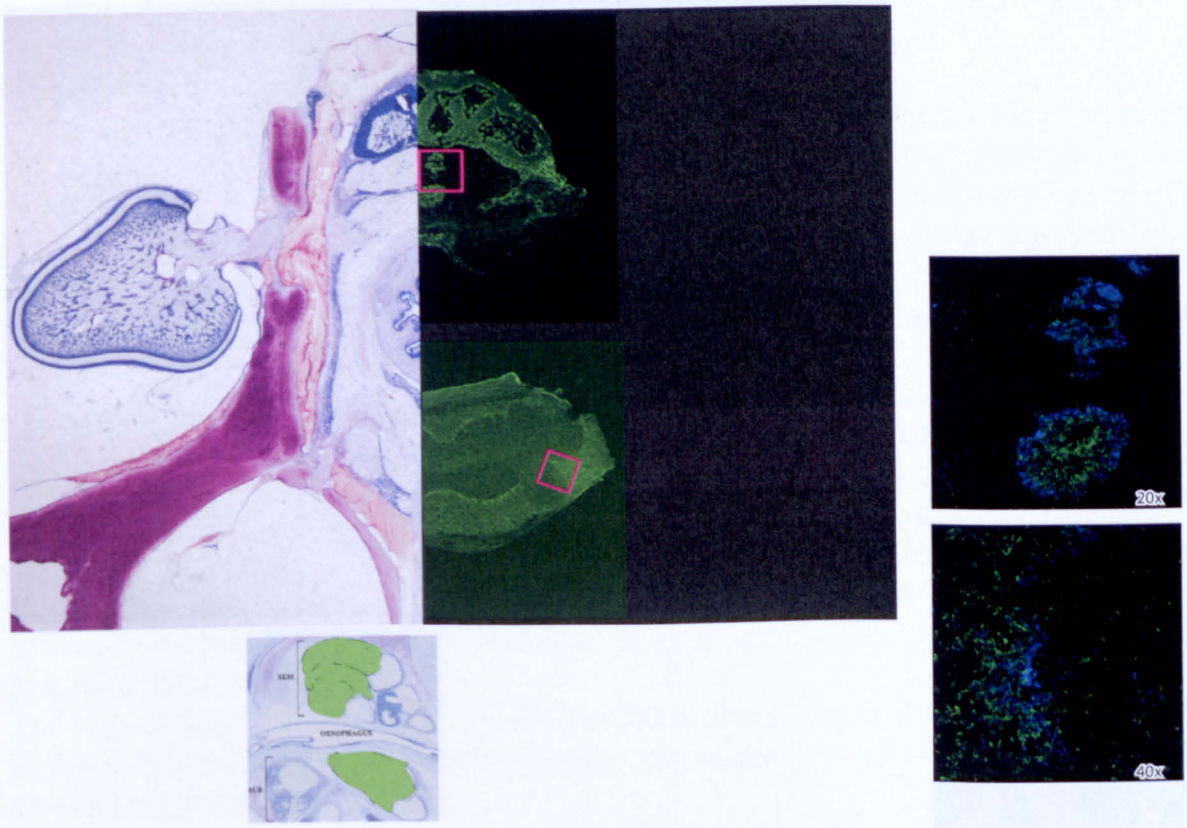


Fig. 4: *Ov-ubi* expression (right panel) is detected in the vertical lobe, sub-vertical lobe, basal lobes (SEM), in the anterior pedal lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 9. For other details see Fig. 1.

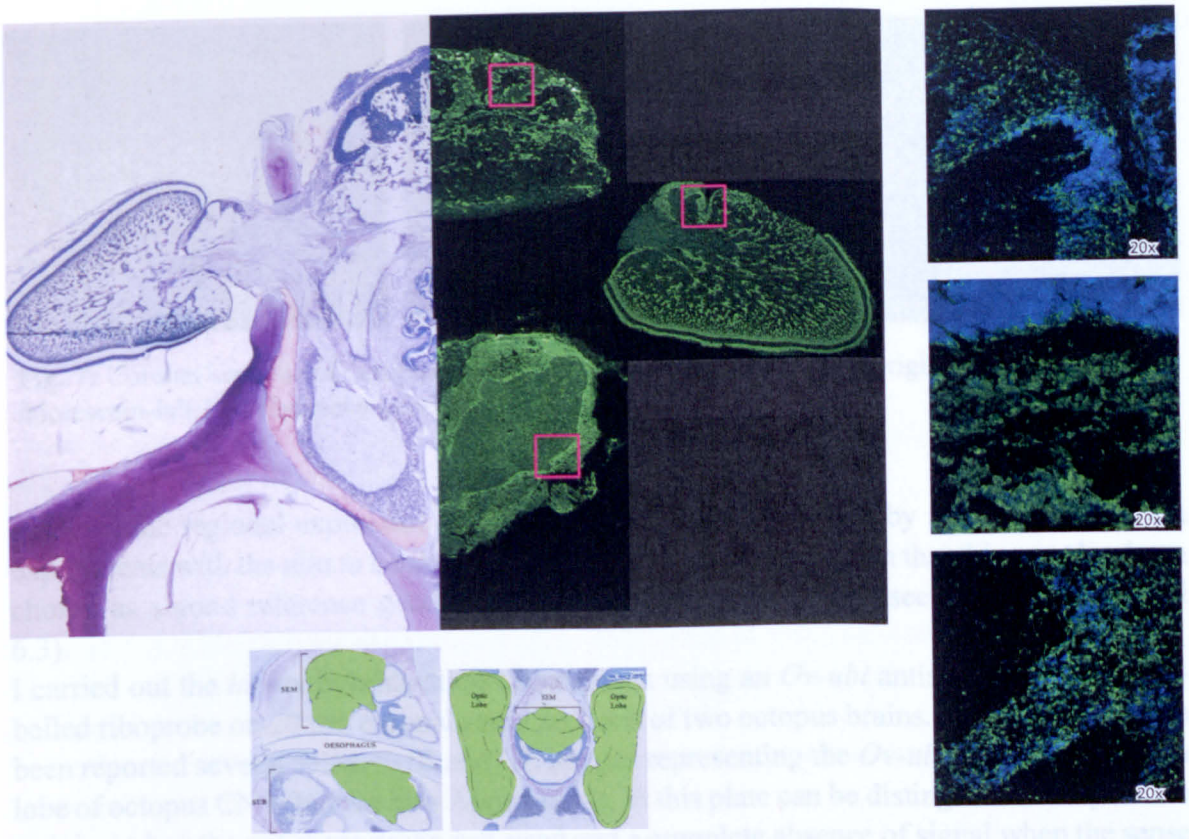


Fig. 5: *Ov-ubi* expression (right panel) is detected in the vertical lobe, sub-vertical lobe, posterior basal lobes (SEM), in the posterior pedal lobe, magnocellular lobe (SUB), in the medulla, inner and outer layer, olfactory lobe and optic gland (OL). For anatomical reference in the left panel see Appendix 3-plate 12. For other details see Fig. 1.

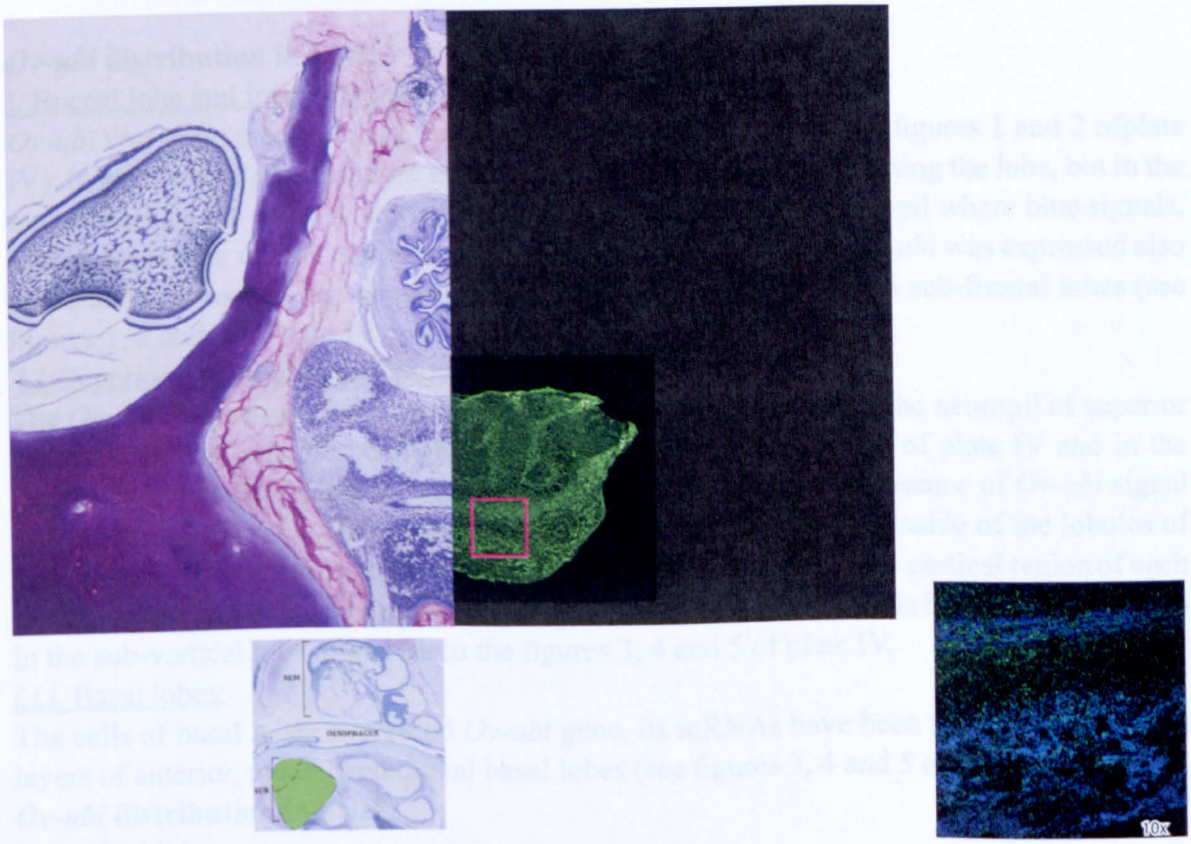


Fig. 6: *Ov-ubi* expression (right panel) is detected in the posterior cromatophore lobe and palliovisceral lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 14. For other details see Fig. 1.

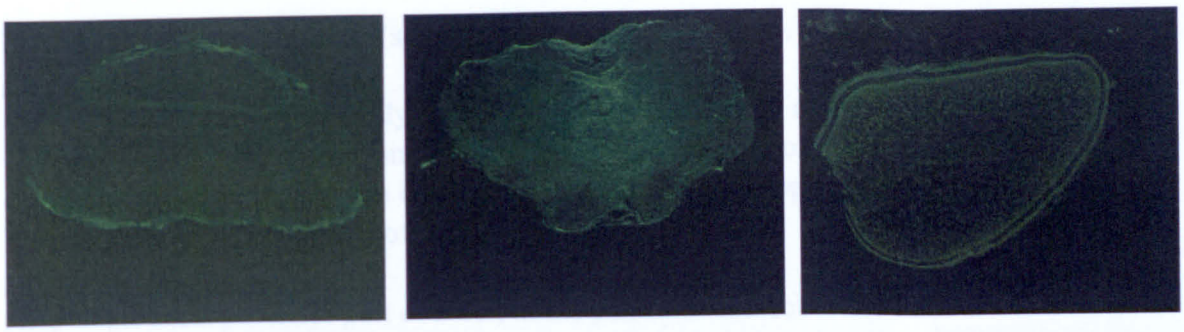


Fig. 7: Coronal sections of octopus brain (SEM, left; SUB, middle; OL, right) after *Ov-ubi* sense fluorescein-labelled riboprobe hybridization.

I studied the regional expression of *Ov-ubi* in the *O. vulgaris* CNS by *in situ* hybridization experiments with the aim to control its ubiquitarious expression given that this gene has been chosen as a good reference gene for real-time qPCR experiments (see paragraphs 6.2 and 6.3).

I carried out the *in situ* hybridization experiments using an *Ov-ubi* antisense fluorescein-labelled riboprobe on 20 µm coronal serial sections of two octopus brains. In the plate IV have been reported several SEM, SUB and OL images representing the *Ov-ubi* expression in each lobe of octopus CNS (but see also Appendix 4). In this plate can be distinguished the positive staining when the antisense probe was used and a complete absence of signal when the sense probe was hybridized (see figure 7). In each lobes of brain masses the cortex was mainly labelled, but in some cases the positive signals can be distinguished also in the neuropil. The magnifications of the lobes of each brain mass are reported in the right panel of figures. The magnification images have been made using the confocal microscope.

***Ov-ubi* distribution in SEM**

i. Buccal lobe and inferior frontal lobe:

Ov-ubi was expressed in the superior and posterior buccal lobe (see figures 1 and 2 ofplate IV), in particular it seemed more abundant in the cellular layer supporting the lobe, but in the magnifications the positive signals appeared present also in the neuropil where blue signals, related to cellular nuclei, are not present (see Appendix 4-plate I). *Ov-ubi* was expressed also in the anterior, medial and posterior part of the inferior frontal and in sub-frontal lobes (see figures 1, 2 and 3 of plate IV).

i.i. Superior frontal lobe and vertical lobe:

The *Ov-ubi* transcripts have been found in the cortical region and in the neuropil of superior frontal lobe. The relative positive signals are shown in the figure 3 of plate IV and in the figures of Appendix 4-plate II, where it is possible to observe the presence of *Ov-ubi* signal in the neuropil. The *Ov-ubi* riboprobe specifically labelled the cells inside of the lobules of vertical lobe, where there are the larger cells of the lobe, but also in the cortical region of each lobule (see figures 4 and 5 of plate IV). Moreover positive labelled cells have been found also in the sub-vertical lobe as shown in the figures 3, 4 and 5 of plate IV.

i.i.i. Basal lobes:

The cells of basal lobes expressed *Ov-ubi* gene, its mRNAs have been found in the cortical layers of anterior, medial and dorsal basal lobes (see figures 3, 4 and 5 of plate IV).

***Ov-ubi* distribution in SUB**

i. Brachial lobe:

The prebrachial and posterior brachial lobes contained cells which expressed *Ov-ubi*, the mRNAs of this gene have been found only in the cortical layer, any positive signals have

been detected in the neuropil (see figures 1 and 2 of plate IV).

ii. Pedal lobes:

Ov-ubi has been ubiquitariously expressed in the pedal lobes: anterior, medial and posterior. This gene was expressed not only in the cortical layer, but also in the neuropil (see figures 3, 4 and 5 of plate IV). Also, the cells of magnocellular lobe appeared to contain *Ov-ubi* mRNAs as shown in the figure 5 of plate IV.

iii. Palliovisceral lobe:

Also in the most posterior part of SUB, in the palliovisceral lobe, has been found the *Ov-ubi* mRNA (see figure 6 of plate IV).

iiii. Chromatophore lobes:

The *Ov-ubi* mRNAs were found in the cells belonging to both anterior (see figure 4 of plate IV) and posterior chromatophore lobes (see figure 6 of plate IV).

***Ov-ubi* distribution in OL**

The *Ov-ubi* transcripts have been abundantly present in SEM and SUB, such as in the optic lobes. This gene seemed expressed in both inner and outer layer of the cortex (see figures 1, 3 and 5 of plate IV), but also in the cell islands of the medulla (see magnifications of figures 1 and 3 of plate IV).

i. Olfactory lobe:

In the figure 5 of plate IV, it is possible to see the presence of *Ov-ubi* mRNA in the cortical layer and in the neuropil of the olfactory lobe.

Plate V-Localization of *ubiquitin C-terminal hydrolase* mRNA in the octopus brain

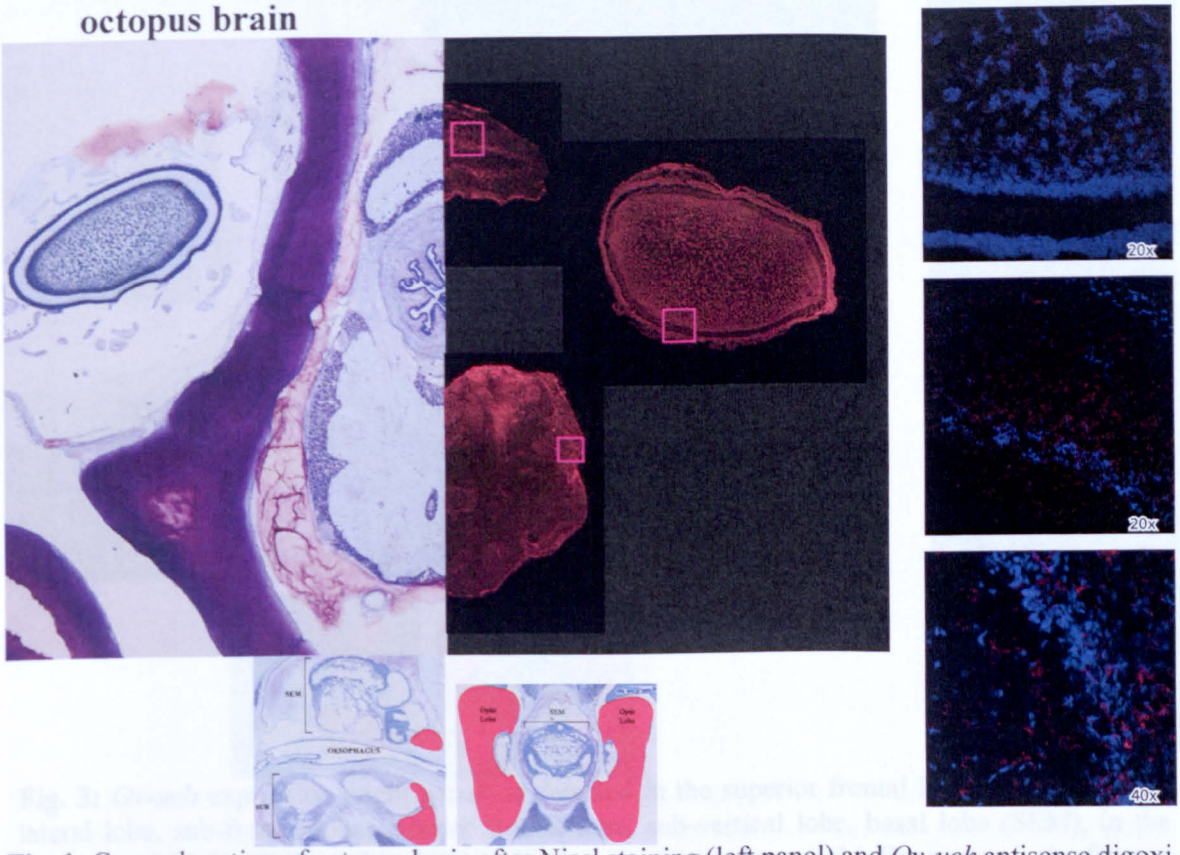


Fig. 1: Coronal section of octopus brain after Nissl staining (left panel) and *Ov-uch* antisense digoxigenin-labelled riboprobe hybridization (right panel). *Ov-uch* expression (right panel) is detected in the superior buccal lobe, inferior frontal lobes (SEM), in the brachial lobe (SUB), in the medulla, inner and outer layer (OL); for anatomical reference in the left panel see Appendix 3-plate 4.
Details (pink square) are presented on the right for each mass (OL, top; SEM, middle; SUB, bottom) with their relative magnification. In the magnification together with the signal of riboprobe is visibile also the cell nuclei with blue staining.

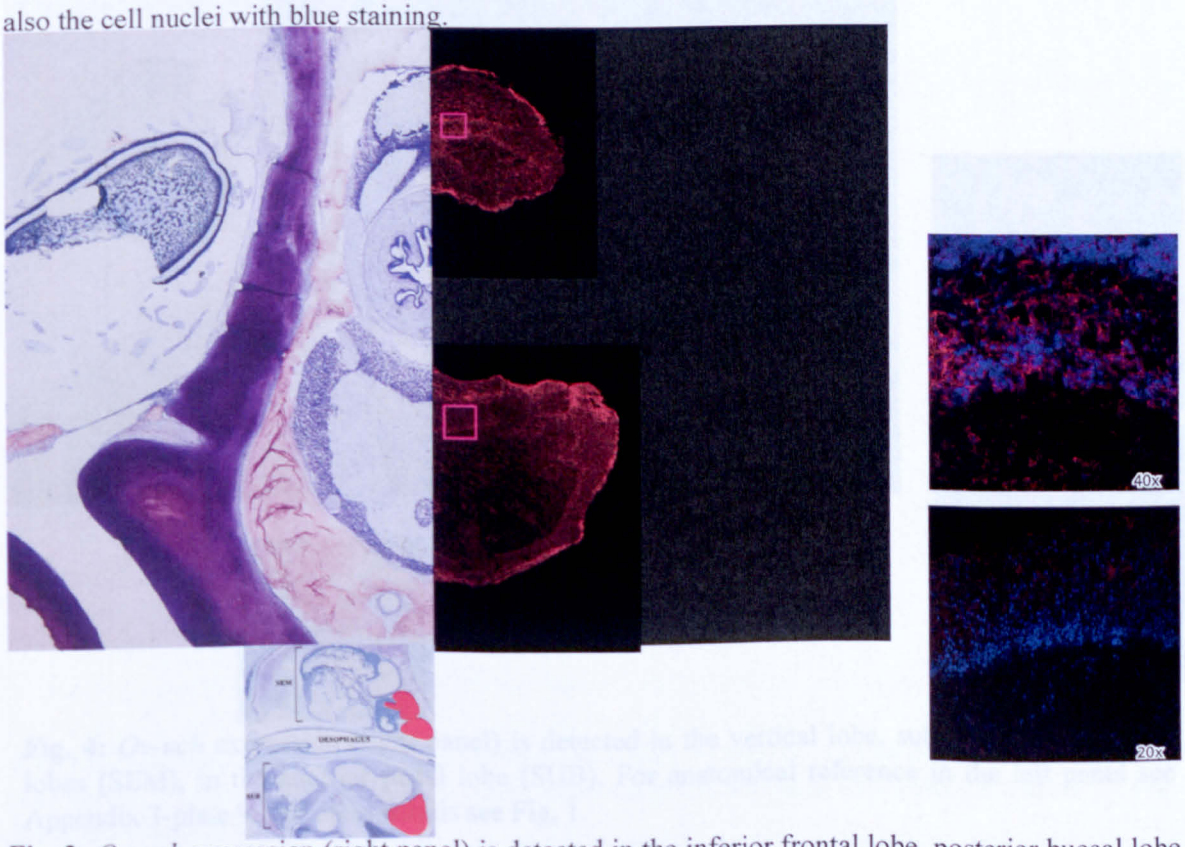


Fig. 2: *Ov-uch* expression (right panel) is detected in the inferior frontal lobe, posterior buccal lobe (SEM) and in the brachial lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 5. For other details see Fig. 1.

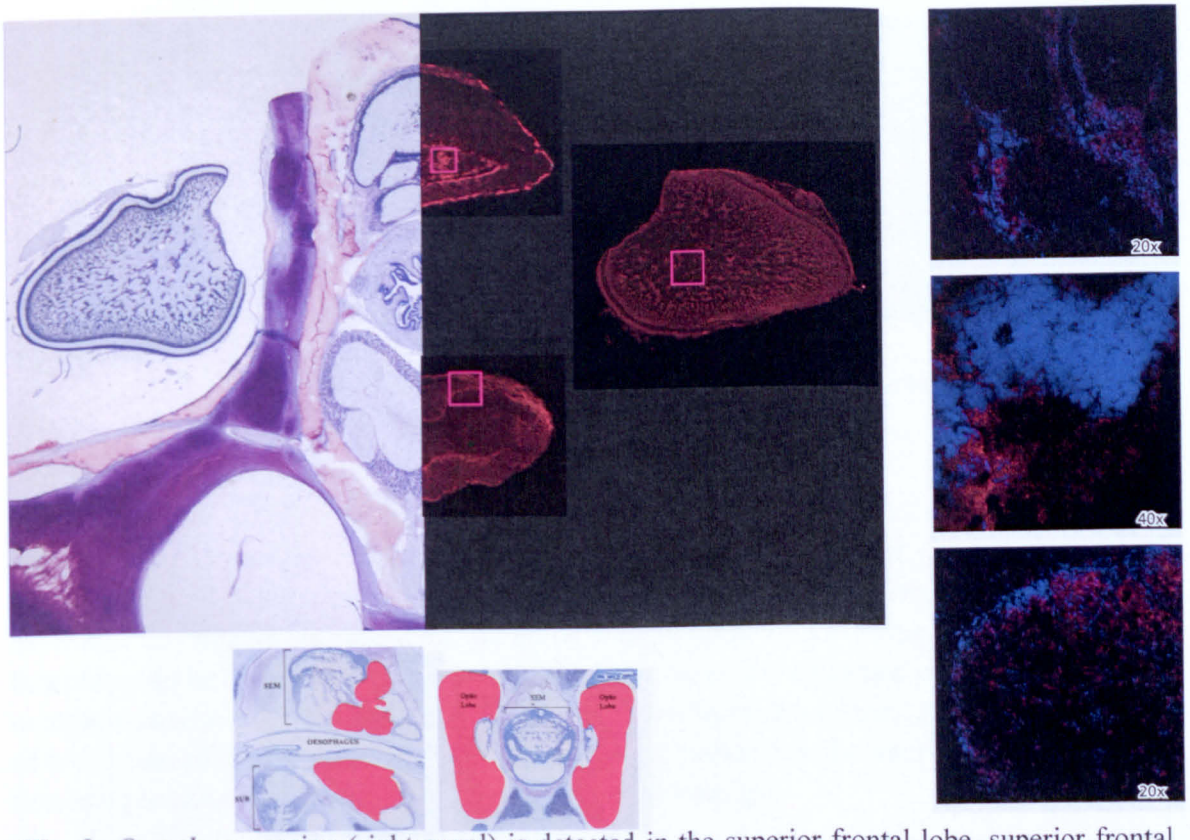


Fig. 3: *Ov-uch* expression (right panel) is detected in the superior frontal lobe, superior frontal lateral lobe, sub-frontal lobe, inferior frontal lobe, sub-vertical lobe, basal lobe (SEM), in the anterior pedal lobe (SUB) and in the medulla, inner and outer layer (OL). For anatomical reference in the left panel see Appendix 3-plate 8. For other details see Fig. 1.

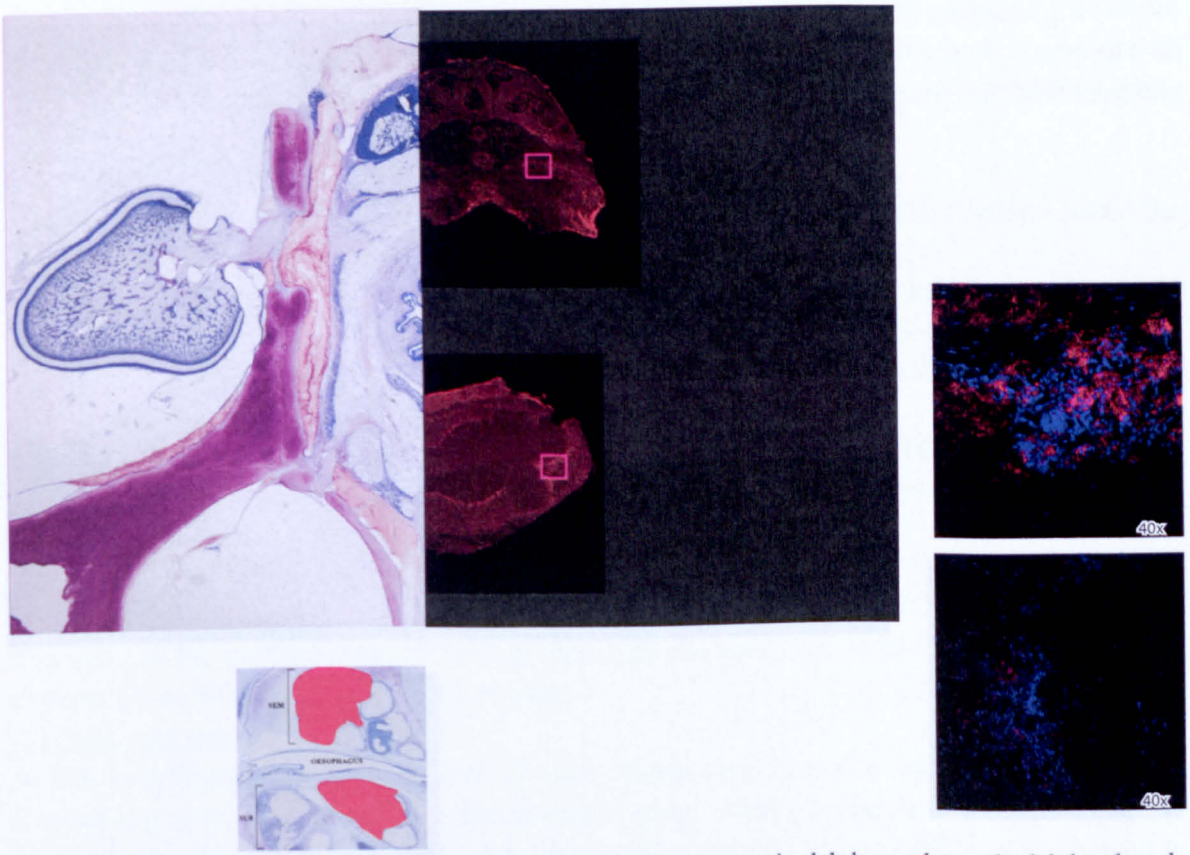


Fig. 4: *Ov-uch* expression (right panel) is detected in the vertical lobe, sub-vertical lobe, basal lobes (SEM), in the anterior pedal lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 9. For other details see Fig. 1.

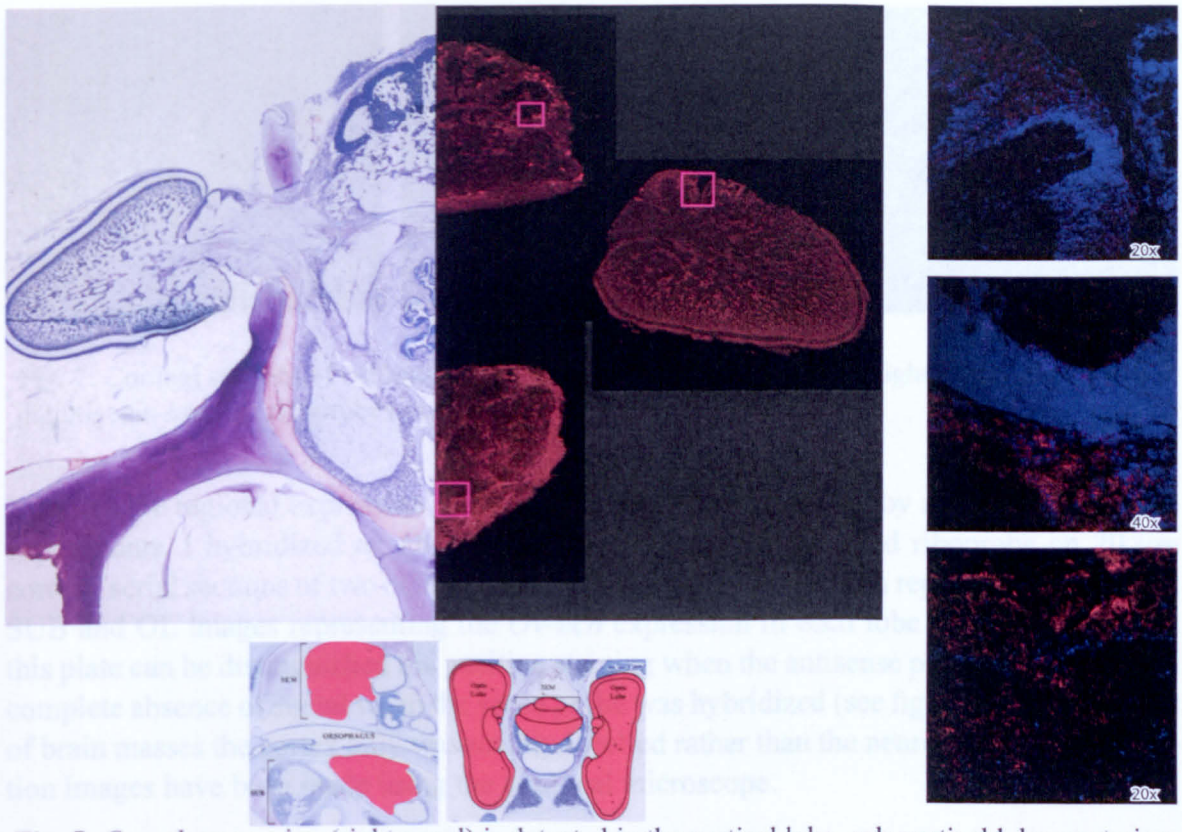


Fig. 5: *Ov-uch* expression (right panel) is detected in the vertical lobe, sub-vertical lobe, posterior basal lobe (SEM), in the posterior pedal lobe (SUB), in the medulla, inner and outer layer, olfactory lobe and optic gland (OL). For anatomical reference in the left panel see Appendix 3-plate 12. For other details see Fig. 1.

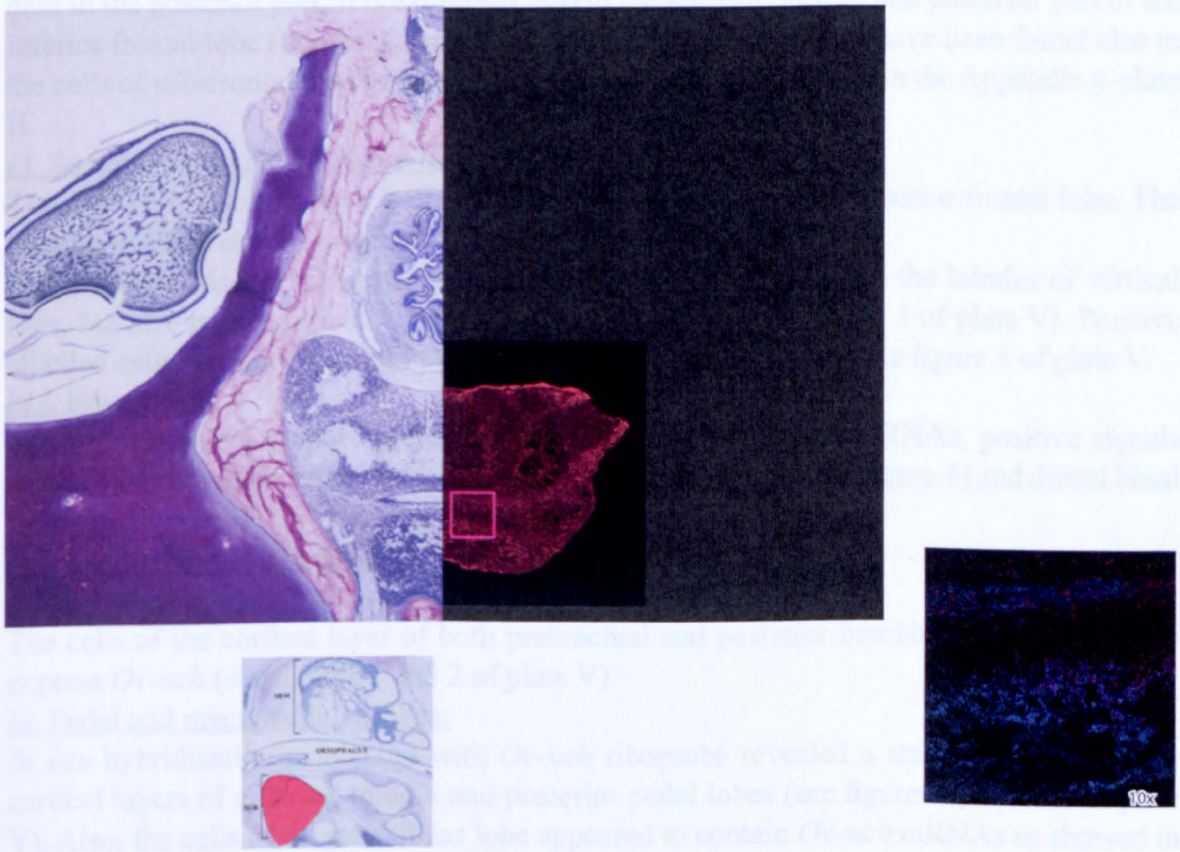


Fig. 6: *Ov-uch* expression (right panel) is detected in the posterior cromatophore lobe, palliovisceral and vasomotor lobes (SUB). For anatomical reference in the left panel see Appendix 3-plate 14. For other details see Fig. 1.

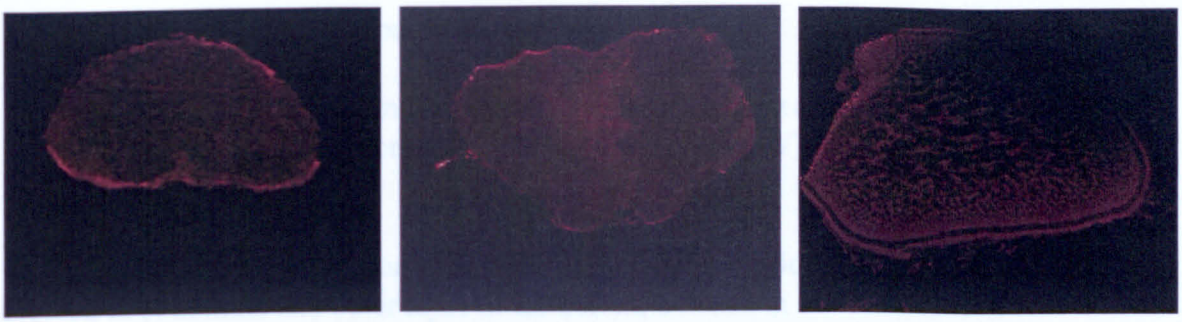


Fig. 7: Coronal sections of octopus brain (SEM, left; SUB, middle; OL, right) after *Ov-uch* sense digoxigenin-labelled riboprobe hybridization.

I studied the regional expression of *Ov-uch* in the *O. vulgaris* CNS by *in situ* hybridization experiments. I hybridized an *Ov-uch* antisense digoxigenin-labelled riboprobe on 20 μm coronal serial sections of two octopus brains. In the plate V have been reported several SEM, SUB and OL images representing the *Ov-uch* expression in each lobe of these masses. In this plate can be distinguished the positive staining when the antisense probe was used and a complete absence of signal when the sense probe was hybridized (see figure 7). In each lobes of brain masses the cortex only was mainly labelled rather than the neuropil. The magnification images have been made using the confocal microscope.

***Ov-uch* distribution in SEM**

i. Buccal lobe and inferior frontal lobe:

Ov-uch is expressed in the cortical region of buccal lobe, in particular it seemed more abundant in the posterior part; it is expressed also in the anterior, medial and posterior part of the inferior frontal lobe (figures 1, 2 and 3 of plate V). Positive signals have been found also in the cells of sub-frontal lobe as reported in the figure 3 of plate V and in the Appendix 4-plate II.

i.i. Superior frontal lobe and vertical lobe:

The *Ov-uch* transcripts have been found in the cortical region of superior frontal lobe. The relative positive signals are shown in the figure 3 of plate V.

The *Ov-uch* antisense riboprobe produced a specific labelling inside the lobules of vertical lobe, but also in the cortical region of each lobule (see figures 4 and 5 of plate V). Positive labelled cells have been found in the sub-vertical lobe as shown in the figure 5 of plate V.

i.i.i. Basal lobes:

The cellular layers supporting the basal lobes contained *Ov-uch* mRNAs, positive signals can be observed in the anterior (see figure 3 of plate V), medial (see figure 4) and dorsal basal lobes (see figure 5).

***Ov-uch* distribution in SUB**

i. Brachial lobe:

The cells of the cortical layer of both prebrachial and posterior brachial lobes appeared to express *Ov-uch* (see figures 1 and 2 of plate V).

i.i. Pedal and magnocellular lobes:

In situ hybridization conducted with *Ov-uch* riboprobe revealed a strong labelling of the cortical layers of anterior, middle and posterior pedal lobes (see figures 3, 4 and 5 of plate V). Also, the cells of magnocellular lobe appeared to contain *Ov-uch* mRNAs as showed in the Appendix 4-plate VI.

i.i.i. Palliovisceral lobe:

As shown in the next to last figure of plate V the *Ov-uch* was expressed, also, in the most

posterior part of SUB, in the palliovisceral lobe.

iiiii. Chromatophore lobes:

The *Ov-uch* mRNAs were found in the cells belonging to both anterior (see Appendix 4-plate V) and posterior chromatophore lobes (see figure 6 of plate V).

***Ov-uch* distribution in OL**

The *Ov-uch* mRNAs have been abundantly found in the optic lobes. This gene seemed expressed in both inner and outer layer of the cortex, but also in the cell islands of the medulla (see figures 1, 3 and 5 of plate V).

i. Olfactory lobe:

In the figure 5 of plate V it is possible to see the presence of *Ov-uch* mRNAs in the cortical layer of olfactory lobe.

Plate VI-Localization of *dopamine transporter* and *tyrosine hydroxylase* mRNA in the octopus brain

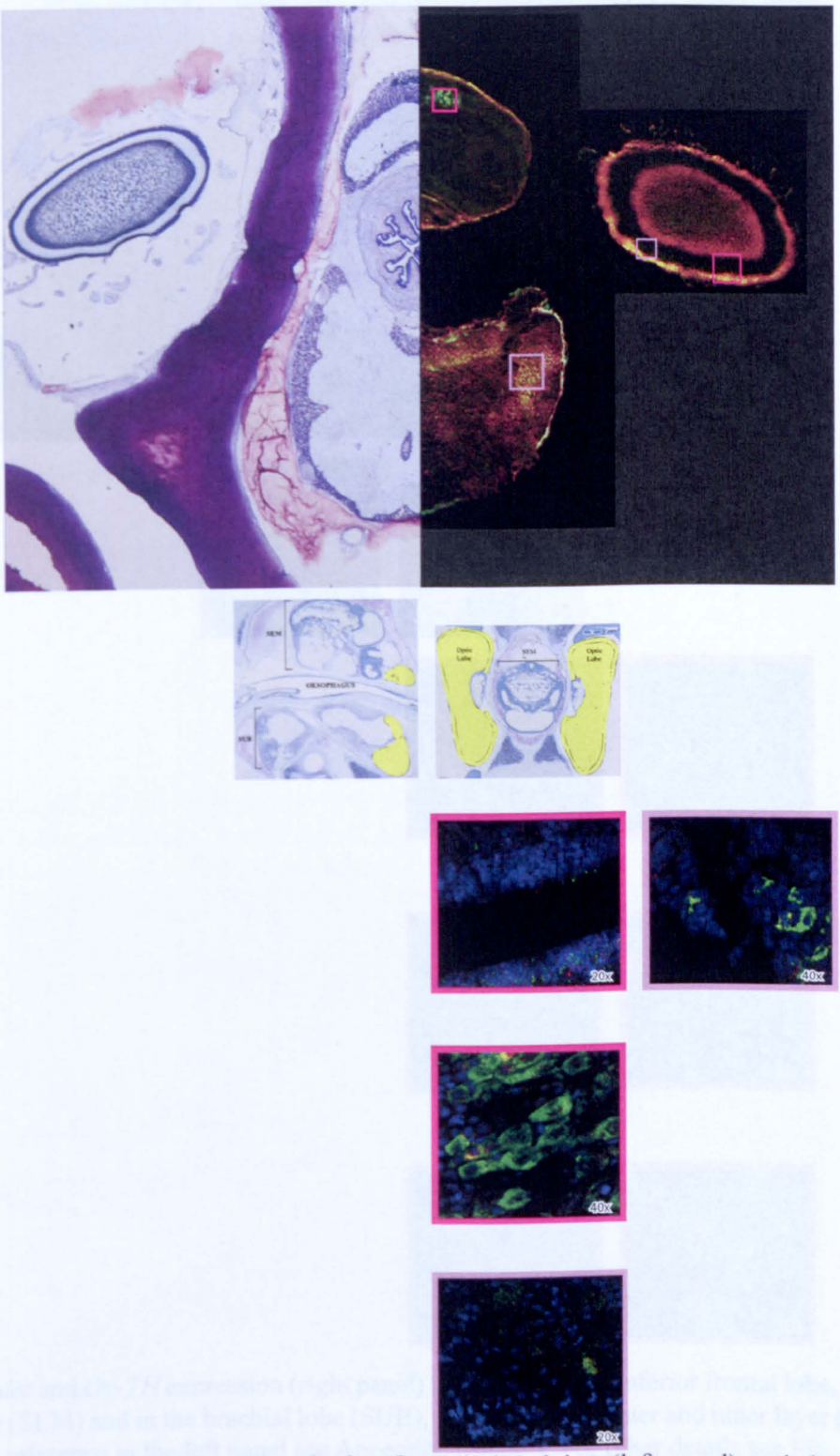


Fig. 1: Coronal section of octopus brain after Nissl staining (left panel) and dat antisense digoxigenin-labelled riboprobe (red signal) together with TH fluorescein-labelled riboprobe (green signal) hybridization (right panel). *Ov-dat* and *Ov-TH* expression (right panel) is detected in the superior buccal lobe, inferior frontal lobe (SEM), in the brachial lobe (SUB), in the medulla, outer and inner layer (OL). For anatomical reference in the left panel see Appendix 3-plate 4.Details (pearl pink and pink squares) are presented below for each mass (OL, top; SEM, middle; SUB, bottom) with their relative magnifications. In the magnification together with the signal of riboprobe is visibile also the cell nuclei with blue staining.

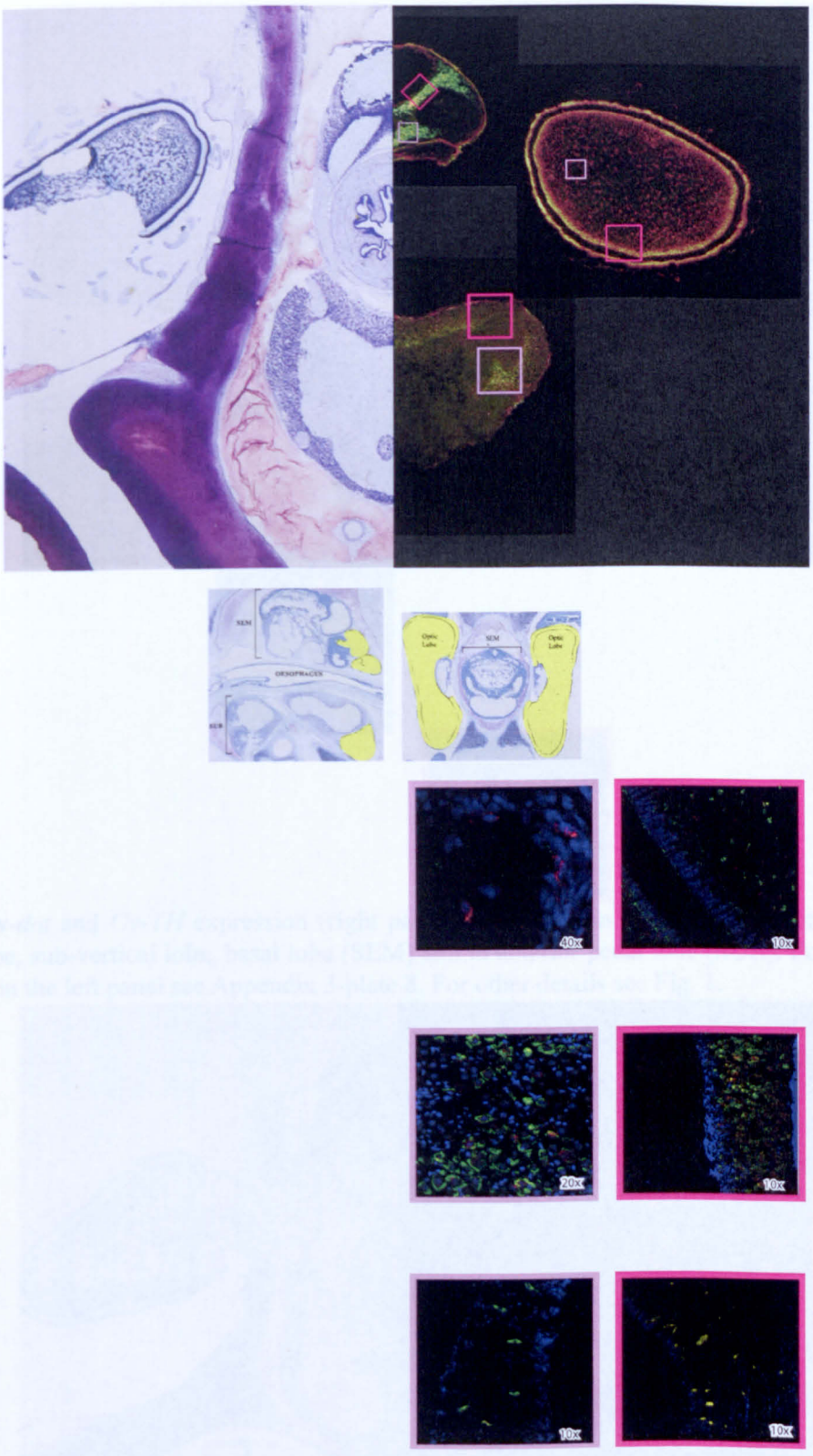


Fig. 2: *Ov-dat* and *Ov-TH* expression (right panel) is detected in the inferior frontal lobe, posterior buccal lobe (SEM) and in the brachial lobe (SUB), in the medulla, outer and inner layer (OL). For anatomical reference in the left panel see Appendix 3-plate 5. For other details see Fig. 1.

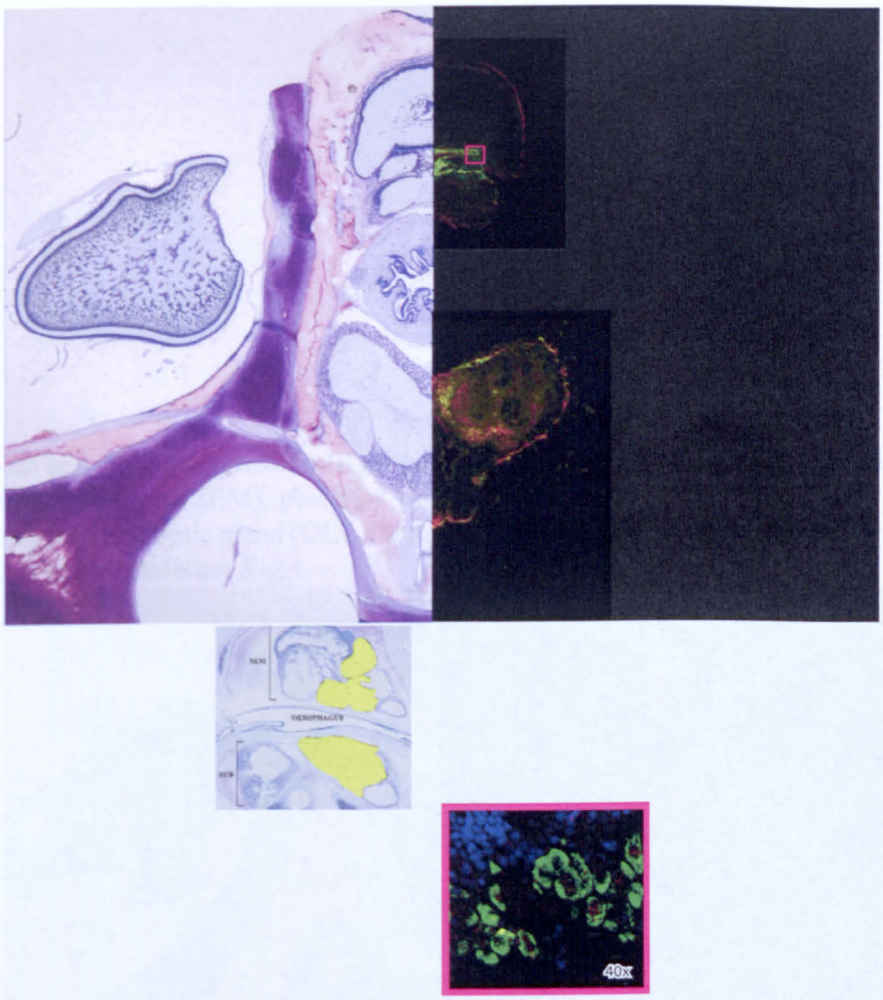
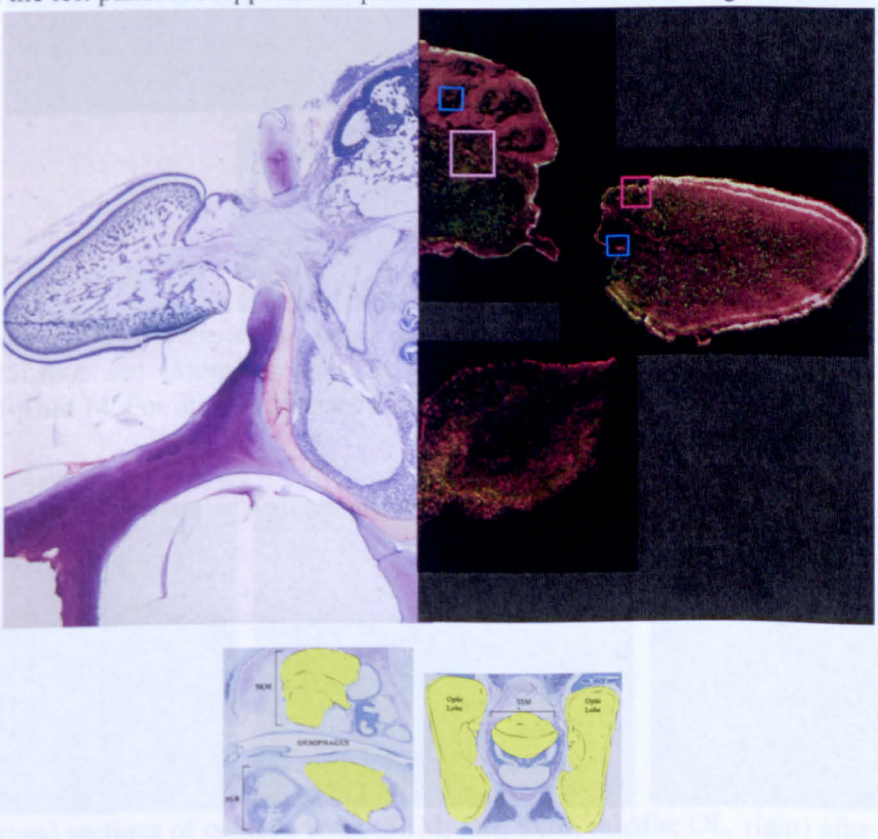


Fig. 3: *Ov-dat* and *Ov-TH* expression (right panel) is detected in the superior frontal lobe, sub-frontal lobe, sub-vertical lobe, basal lobe (SEM) and in anterior pedal lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 8. For other details see Fig. 1.



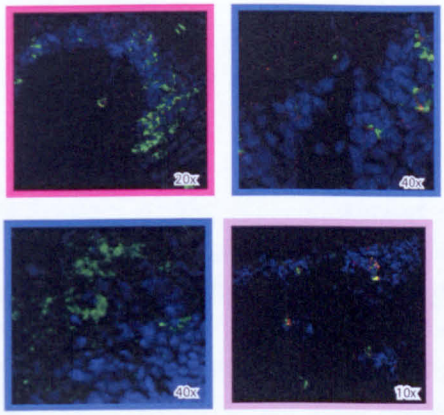


Fig. 4: *Ov-dat* and *Ov-TH* expression (right panel) is detected in the vertical lobe, sub-vertical lobe, posterior basal lobe (SEM), posterior pedal lobe (SUB), in the medulla, inner and outer layer, olfactory lobe and optic gland (OL). For anatomical reference in the left panel see Appendix 3-plate 12. For other details see Fig.1.

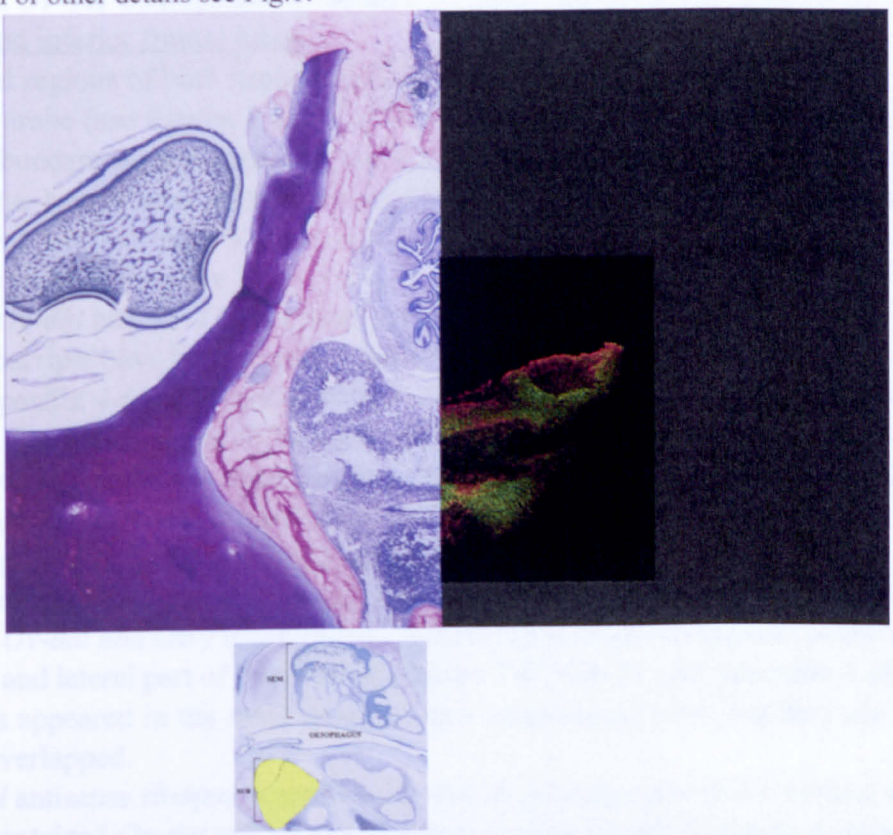


Fig. 5: *Ov-dat* and *Ov-TH* expression (right panel) is detected in the posterior cromatophore lobe, palliovisceral lobe and vasomotor lobe (SUB). For anatomical reference in the left panel see Appendix 3-plate 14. For other details see Fig. 1.

Fig. 6: Coronal sections of octopus brain (SEM, left; SUB, middle; OL, right) after *Ov-dat* and *Ov-TH* sense labelled riboprobes hybridization.

[2]

[2] The localization of *Ov-dat* and *Ov-TH* in the octopus central nervous system was in detail observed by in situ hybridization on 20 μm coronal serial sections of four octopus brains using two antisense riboprobes. First one was synthetized incorporating nucleotides conjugated with digoxigenin and the other one contained nucleotides conjugated with fluorescein. Several coronal sections of supra-oesophageal mass , sub-oesophageal mass and optic lobe are collected in the plate IV. In the figure 6 can be observed a complete absence of signal when the sense probe was used like a specificity probe control. The magnification presented have been made using the confocal microscope.

***Ov-dat* and *Ov-TH* distribution in SEM**

i. Buccal and inferior frontal lobes:

The cortical regions of both superior and the posterior buccal lobes appeared to be labelled by *Ov-dat* probe (see figures 1 and 2 of plate IV, but see also Appendix 4-plate I), even if the more abundant signals appeared in the posterior buccal lobe. Also *Ov-TH* mRNA was present in the buccal lobe, where it is highly expressed in both superior and posterior parts (see figures 1 and 2 of plate VI, see alco Appendix 4-plate I). Looking at the magnification in the right panel of the plate VI, it is possible to deduce that the few cells of buccal lobe co-expressed *Ov-dat* and *Ov-TH*, but many other expressed only *Ov-TH*.

Ov-dat transcripts have been found in the inferior frontal lobe (see figures 1 and 2 of plate VI and Appendix 4-plate I) and in sub-frontal lobe (see figure 3 of plate VI and Appendix 4-plate II). *Ov-TH* appeared to be more abundant than *Ov-dat* in the inferior frontal lobe, instead it seems to have the same distribution of *Ov-dat* in the sub-frontal lobe. The merged images suggested that in this lobe the majority of cells co-expressed together the genes.

i.i. Superior frontal , vertical and sub-vertical lobes:

In situ hybridization showed moderate labelling of superior frontal lobe's cortical regions using both *Ov-dat* and *Ov-TH* riboprobes. It could be possible distinguish positive signals in the medial and lateral part of this lobe (see figure 3 of plate IV and Appendix 4-plate II). The two signals appeared in the same area of supra-oesophageal mass, but they are not always perfectly overlapped.

The *Ov-TH* antisense riboprobe produced a specific labelling also in the vertical lobe, where few cells contained *Ov-dat* mRNA (see figure 4 of plate VI and Appendix 4-plateIII).

Strongly labelled cells were found in the sub-vertical lobe where are present several islands of cells which seemed to contain both *Ov-dat* and *Ov-TH* mRNA (see figure 4 of plate VI and Appendix 4-plateIII).

i.i.i. Basal lobes:

Throughout the basal lobes, the brain cortex showed a slight labelling with *Ov-dat* and *Ov-TH* riboprobes, with the exception of anterior basal lobe and the dorsal basal lobe, whose labelling appeared more intense (see figures 3 and 4 of plate VI and plates II and III of Appendix 4). Two genes were complete overlapped in some regions and in some other ones they were expressed in different cells and with very different abundance. In particular, there was a strong prevalence of cells expressing *Ov-TH*.

***Ov-dat* and *Ov-TH* distribution in SUB**

i. Brachial lobes:

In situ hybridization revealed a strong labelling of both pre- and posterior brachial lobes

using the two probes *Ov-dat* and *Ov-TH* (see figures 1 and 2 of plate VI and plate IV of Appendix 4). These genes appeared co-expressed in the cells of these lobes, the magnifications reported in the Appendix 4 confirmed this conclusion.

i.i. Pedal and magnocellular lobes:

The cortical layers of pedal lobes showed to contain cells expressing both *Ov-dat* and *Ov-TH.* They appeared expressed everywhere in the pedal lobes: in anterior, middle and posterior parts (see figures 3 and 4 of plate VI and plates V and VI of Appendix 4). The cells present in the magnocellular lobe appeared to express both genes (see Appendix 4-plate V).

i.i.i. Palliovisceral and vasomotor lobe:

The cells belonged to palliovisceral and vasomotor lobes appeared to contain both *Ov-dat* and *Ov-TH* mRNAs (figure 5 of plate VI and Appendix 4-plate VI).

i.i.i.i. Chromatophore lobes:

The chromatophre lobes in the anterior part and in the posterior part of sub-oesophageal mass expressed *Ov-dat* and *Ov-TH* (see figures 2 and 5 of plate VI).

Ov-dat and _Ov-TH_ distribution in OL

In the cells of optic lobes have been found both *Ov-dat* and *Ov-TH* transcripts. These genes appeared more expressed in the outer layer of cortex than in the inner one (see figures 1, 2 and 4 of plate VI). They were expressed also in the islands of cells present in the medulla, but not always they were co-expressed (see figure 4 of plate VI and plate VII of Appendix 4).

i. Olfactory lobe and optic gland:

The cortical regions of both the olfactory lobe and the optic gland were labelled by the *Ov-dat* and *Ov-TH* riboprobes (see figure 4 of plate VI).

6.4 Discussion

The aim of the present study was to investigate the distribution of target (*Ov-creb, Ov-dat, Ov-TH and Ov-stm*) and reference gene (*α-tubulin, Ov-ubi*) transcripts in the octopus brain by mean of *in situ* hybridization.

This with the aim of providing information of the differential expression of the genes, target for this thesis, within the brain of the octopus and possibly within given lobes. In my view this may help in the analysis of the results obtained in other experiments I carried out during my PhD by providing insights that may be utilized to correlate neuro-anatomical, functional and molecular data within a common framework.

In situ hybridization experiments are not new to octopuses (i.e. oct-GnRH, Iwakoshi-Ukena *et al.*, 2004; calretinin, Altobelli and Cimini, 2007; tachykinins, Kanda *et al.*, 2003; oct-GnRHR, Kanda *et al.*, 2006; cephalotocin and octopressin, Takuwa-Kuroda *et al.*, 2003) but any information are available about expression of target genes of my thesis in the central nervous system of octopuses. Instead, the expression of genes of interest for this thesis has been investigated in the brain of vertebrates (e.g. for uch see Kurihara *et al.*, 2001; for stathmin see Shumyatsky *et al.*, 2002; for dat see Lindblom *et al.*, 2006; for TH see Robinson *et al.*, 2007; for creb see Han *et al.*, 2008), and invertebrates (e.g for uch see Hegde *et al.*, 1997; for stathmin see Ozon *et al.*, 2002; for creb see Ribeiro *et al.*, 2003; for dat and TH see McDonald *et al.*, 2006)

The results of analysis carried out for this thesis are summarized in the table 6.2 and indicate that these mRNAs are present in SEM, SUB and OL.

The examination of coronal brain sections has shown the expression of genes of interest in several areas of the octopus brain. The staining intensity was variable among the different examined lobes.

tubA and *Ov-ubi,* here selected as reference genes resulted to be effectively ubiquitarius. They were expressed in all analyzed lobes of three octopus brain masses, in particular *tubA* has been found in the cortical regions of each brain lobe, whereas *Ov-ubi* was expressed not only in the cortical regions but also in the regions belonging to neuropil of buccal and superior frontal lobe (in the SEM), of pedal lobes (in the SUB) and of olfactory lobe (in the OL).

Among the target genes of this study, *Ov-creb* and *Ov-uch* mRNAs were found in the cytoplasm of cells belonging to the cortical layer of each lobe of octopus CNS, like the two ubiquitously expressed genes.

Also the transcripts of *Ov-stm* have been found in all observed lobes of octopus brain, but their distribution is not limited to the lobes' cortical regions, but also in the neuropil of superior buccal and superior frontal lobes *Ov-stm* is expressed.

Table 6.2: Summary of localization of target genes' (*Ov-creb, Ov-dat, Ov-TH, Ov-stm* and *Ov-uch*) and reference genes' (*tubA, Ov-ubi*) transcripts in the cortical region (CR) or neuropil (N) of octopus brain lobes. Highlighted in green are regions where gene transcripts were found, in red regions where they are absent

Brain lobes	***Ov-creb***		***Ov-dat***		***Ov-TH***		***Ov-stm***		***Ov-uch***		***α-tub***		***Ov-ubi***	
	CR	N	CR	N	CR	N	CR	N	CR	N	CR	N	CR	N
SEM														
Superior buccal	√		√		√		√	√	√		√		√	√
Posterior buccal	√		√		√		√		√		√		√	√
Inferior frontal	√		√		√		√		√		√		√	
Sub-frontal	√		√		√		√		√		√		√	
Superior frontal	√		√		√		√	√	√		√		√	√
Vertical	√		√		√		√		√		√		√	
Sub-vertical	√		√		√		√		√		√		√	
Anterior basal	√		√		√		√		√		√		√	
Medial basal	√		√		√		√		√		√		√	
Dorsal basal	√		√		√		√		√		√		√	
SUB														
Prebrachial	√		√		√		√		√		√		√	
Postbrachial	√		√		√		√		√		√		√	
Anterior pedal	√		√		√		√		√		√		√	√
Middle pedal	√		√		√		√		√		√		√	√
Posterior pedal	√		√		√		√		√		√		√	√
Chromatophore	√		√		√		√		√		√		√	
Magnocellular	√		√				√		√		√		√	
Palliovisceral	√		√		√		√		√		√		√	
OL														
Optic	√		√		√		√		√		√		√	
Olfactory	√		√		√		√		√		√		√	√

The *Ov-TH* and *Ov-dat* transcript distribution has been studied by double *in situ* hybridization experiments with the aim to identify the cells expressing both genes (e.g. dopaminergic neuron) or the other ones that contain only *Ov-TH* (e.g. noradrenergic neuron).

Ov-dat is ubiquitously distributed, it is expressed in the call bodies of neurons of every lobes of octopus CNS. It has been found in more moderate quantities with respect to *Ov-TH. Ov-TH* is more highly expressed than *Ov-dat* in all the other lobes analyzed (except the magnocellular one).

The data related to the fine distribution and the co-localization of *Ov-TH* and *Ov-dat* transcripts are reported in the table 6.3. The results of *in situ* experiments, in particular the presence of two transcripts in the same cells, allowed me to define the distribution of dopaminergic and noradrenergic neurons in the octopus CNS (see table 6.3). These data complete, reinforce and, in some cases, contrast with the neurotransmitters distribution reviewed by J.B. Messenger (1996; table 6.3). These data were the results of experiments

that applied different technology such as thin-layer chromatography, HPLC, enzymatic and fluorometric analysis of carefully dissected lobes of the brain.

As reported in the table 6.3 the *in situ* results suggested that in every octopus lobe of CNS are present both dopaminergic and noradrenalinergic neurons, except in the anterior and middle pedal lobe and in magnocellular lobe where only dopaminergic ones have been found.

In the lobes where Messenger (1996) reported both catecholamines, the *in situ* experiments suggested the presence of cells expressing both *Ov-TH* and *Ov-dat* mRNAs and some others containing only *Ov-TH* transcripts confirming the presence of dopaminergic and noradrenergic neurons. These two types of neurons have been found also in the major number of lobes considered to contain potentially dopamine and/or noradrenaline (DA/NA), except in three lobes of SUB which contained only dopaminergic neurons.

Table 6.3: In this table are summarized the results of double *in situ* with *Ov-dat* and *Ov-TH* probes and the identification of dopaminergic (DA) and noradrenergic (NA) neurons. These results are compared with previous available knowledge (Messenger, 1996) about the distribution of transmitters/modulators in the brain (DA; NA: dopamine and noradrenaline; ?: dopamine and/ or noradrenaline, dubious results).

Brain lobes	***Ov-dat* and *Ov-TH* *in situ* results**		**Messenger 1996**
Superior buccal	DA	NA	DA; NA
Posterior buccal	DA	NA	DA; NA
Inferior frontal	DA	NA	DA; NA
Sub-frontal	DA	NA	DA/NA
Superior frontal	DA	NA	DA; NA
Vertical	DA	NA	DA; NA
Sub-vertical	DA	NA	?
Anterior basal	DA	NA	?
Medial basal	DA	NA	?
Dorsal basal	DA	NA	?
Prebrachial	DA	NA	DA; NA
Postbrachial	DA	NA	DA; NA
Anterior pedal	DA		?
Middle pedal	DA		?
Posterior pedal	DA	NA	?
Anterior chromatophore	DA	NA	?
Posterior chromatophore	DA	NA	DA; NA
Magnocellular	DA		?
Palliovisceral	DA	NA	?
Optic	DA	NA	DA; NA
Olfactory	DA	NA	?

All analyzed genes are present in lobes of both the SEM and SUB of octopus brain. That means either sensory neurons, mainly belonging to the SEM, or mo-

tor neurons, mainly belonging to the SUB, express the target genes of this study. Moreover, the knowledge of both distribution of these mRNAs containing neurons and differential staining of the different lobes give information about the role played by these target genes in the control of specific neuronal processes (e.g.: feeding sequence, tactile and visual memory systems, arm movement system). For example, the presence of every studied gene in the superior buccal lobe suggested their potential role in the control of neuromodulation and neurotransmission in the feeding sequence (Young, 1971). These hypothesis is confirmed for CREB by studies conducted on *Lymnea stagnalis* that demonstrate the importance of this molecule in the feeding behaviour and show its activation in the ganglia containing neurons belonging to the feeding circuitry (Ribiero et al., 2003). The distribution in the basal lobes indicated their potential involvement in the regulation of the motor centre controlling swimming, respiration and other actions. The involvement of dat in the motor control has been studied in *C. elegans* where the changes in the expression of dat in the cephalic neurons has been related to the regulation of locomotion responses (McDonald et al., 2007). In vertebrate, both *dat* and *TH* have been expressed in the neurons of basal ganglia system known to be involved in the planning and modulation of movement pathways (for dat see Weiss et al., 2007; for TH see Robinson et al., 2007). *O. vulgaris* has separate memory systems for touch and vision, my data, although preliminary, suggest a potential role of the genes I considered target for this study involved in the modulation of both visual and tactile memory system. One of them, *creb*, is known to be activated in the vertebrate limbic system demonstrating its important role in the formation of LTM in response to different forms of learning (e.g. fear conditioning and social learning; for review see Silva et al., 1998; Josselyn and Nguyen, 2005). Also *stathmin* resulted expressed in response to innate or learned fear in limbic system neurons. In analogy to what is known for *Stathmin* and *Creb* activation in vertebrate neurons, I suppose that they might be involved also in the regulation of visual and/or tactive memory in the octopus The optic lobes are centres for memory storage (e.g. Young, 1962), the strong distribution of all analyzed target genes in this brain mass indicated a possible involvement in the memory storage of visual and tactile experiences. A confirmation of this hypothesis for the gene *TH*, is given by studies conducted in the *D. melanogaster* (Neckameyer, 1998). This gene is involved in learning processes and is prevalently expressed in mushroom bodies, a brain area known to play an important role in the memory storage, such as octopus optic lobes (Krashes et al., 2007). The brachial lobes are small relative to the total brain volume, in octopus they are evidently related to the usage of the arms (Young, 1971). The wide distribution of target gene mRNA expressing cell bodies in the brachial lobes suggested that they may affect neuronal control of the operation of the arms. The known involvement of magnocellular lobes in the dynamics reactions and, in general, in the defensive actions let to think that the tar-

get genes expression in these lobes could be related to the regulation of these actions. The knowledge of the regions where the target genes are expressed in the octopus brain may eventually provide a way to find changes induced in response to specific behavioural experiences. This study represents just the starting point: it is hopefully expected that patterns of target gene distribution can be further analyzed in response to behavioural experiences to deduce the genes' involvement in learning and memory processes.

CHAPTER 6

FEAR CONDITIONING IN *OCTOPUS VULGARIS*: THE ROLE OF CREB PHOSPHORYLATION

Nine *O. vulgaris* were utilized in experiments aimed to test whether cAMP response element-binding protein (CREB) is activated in response to fear conditioning as suggested to occur in other taxa (e.g. Silva *et al.*, 1998; Kandel, 2001; Tully *et al.*, 2003; Won and Silva, 2008; Benito and Barco, 2010; Radulovic and Tronson, 2010).

6.1 Materials and methods

6.1.1 Subjects

Octopuses of both sexes (body size: 200 - 350 g) were acclimated[1] and randomly assigned to three experimental groups. Naïve animals (Naive, N = 3), were sacrificed about seven days after capture and served as control. Trained octopuses (Trained, N = 3) were sacrificed one hour after the end of a training session (for details see appendix 2). In addition, three more animals were sacrificed one hour after the end of the testing session of fear conditioning (Tested).

6.1.2 Samples

After dissection the four parts of the brain (the left and right OLs, SEM, SUB)[1]were immediately placed in sterile tubes containing 1 ml of RIPA buffer[2]. Samples were stored at -80°C until further processing.

1 For details see Appendix 1 - Animals.

2 RIPA buffer: 50mM TRIS HCl pH 7.8, 150mM NaCl, 10mM EDTA, 0.5% NP 40, 0.1% Sodium deoxycholate, 0.5% Triton X-100, 1mM PMSF, 1mM DTT, protease and phosphatase inhibitors (Roche, Indianapolis, IN).

6.1.3 Protein extraction and quantification

Frozen samples were homogenized with Ultra turrax T25 homogenizer (Janke & Kunkle, Staufen, Germany) and incubated on wet ice for 30 minutes. Samples were then centrifuged at 15000 rpm for 30 minutes at 4°C. The supernatant, containing proteins, was stored at -80°C.

Protein concentrations were estimated using the Bio-Rad Protein Assay (Bio-rad, Melville, NY, USA). This method involves the addition of an acidic dye to protein solution, and subsequent measurement at 595 nm with a spectrophotometer. Comparison to a standard curve, using known concentrations of Bovine Serum Albumin (BSA) at six different dilutions, provided a relative measurement of each protein sample concentration.

6.1.4 CREB antibodies

Custom antibodies against *O. vulgaris* CREB were produced for these experiments by Sigma Genosys using as epitope a peptidic sequence of 12 aminoacids (80-RRPSYRKILNEL-91) contained in the KID domain of octopus CREB (see also paragraph 4.3.1). As the OvAb-CREB antibody was produced using the epitope containing the non-phosphorylated form of Ser-83, while OvAb-pCREB antibody was made with the epitope whit the phosphorylated Ser-83.

6.1.5 Western blotting

Protein sample (8.0 µg) were boiled for 5 minutes in the presence of 1X NuPage LDS Sample buffer and 1X Reducing Agent. Samples were run on NuPage 4-12% Bis-Tris Mini Gels (Invitrogen, Paisley, UK) at constant voltage (150 V). After electrophoresis the proteins were transferred to nitrocellulose membrane Immobilon-P (Millipore, Bedford, MA) using 1X Tris-Glycine Transfer buffer[3]. Then, the nitrocellulose membrane was incubated in Blocking buffer[4] for 1 h at room temperature. Membranes were incubated overnight at 4°C with one of the following primary antibodies:

OvAb-CREB (rabbit custom polyclonal antibody; dilution 1:4000, Sigma Genosys, Cambridge, UK); or:

OvAb-pCREB (rabbit custom polyclonal antibody; dilution 1: 4000, Sigma Genosys, Cambridge, UK).

Ab-Actin (polyclonal antibody, diluted 1:10000, Sigma-Aldrich, Saint Louis, Missouri) was also added.

3 25 mM Tris, 190 mM glycine, 0.1% SDS, 20% Methanol.

4 5% powder milk in TTBS:150 mM NaCl, 10 mM Tris pH 7.5, 0.05% Tween 20.

Membranes were then washed three times with TTBS and incubated for 1 hour at room temperature with the horseradish peroxidase-conjugated secondary antibody (1:10000, goat anti-rabbit IgG, Sigma-Aldrich) in Blocking buffer. The membranes were washed again three times with TTBS. Protein bands were visualized using the ECL Plus Western blotting Detection Reagents (Amersham, Buckinghamshire, UK) using the Fluor-S MultiImager (Biorad, Hercules, CA).

Antibody signal from each stained membrane was acquired, the signal analyzed and quantified using the ImageQuant TL software (Amersham Biosciences, Piscataway, NJ).

Differences in the amount of phosphorylated CREB between groups were analysed using analysis of variance (ANOVA). The level of significance was set at $P < 0.05$. SPSS was used for statistical analysis.

6.2 Results

6.2.1 Specificity of OvCREB antibodies

A single band of about 33kDa was detected by OvAb-CREB antibody in homogenates of the different masses of the octopus brain (fig 6.1). On the other hand when using the phosphorylated form produced against the same epitope of KID domain and containing the phosphorylated form of Ser-83 (figure 6.1a), both not-phosphorylated (npCREB; 33kDa) and, although with a weaker band, phosphorylated CREB (pCREB) were revealed.

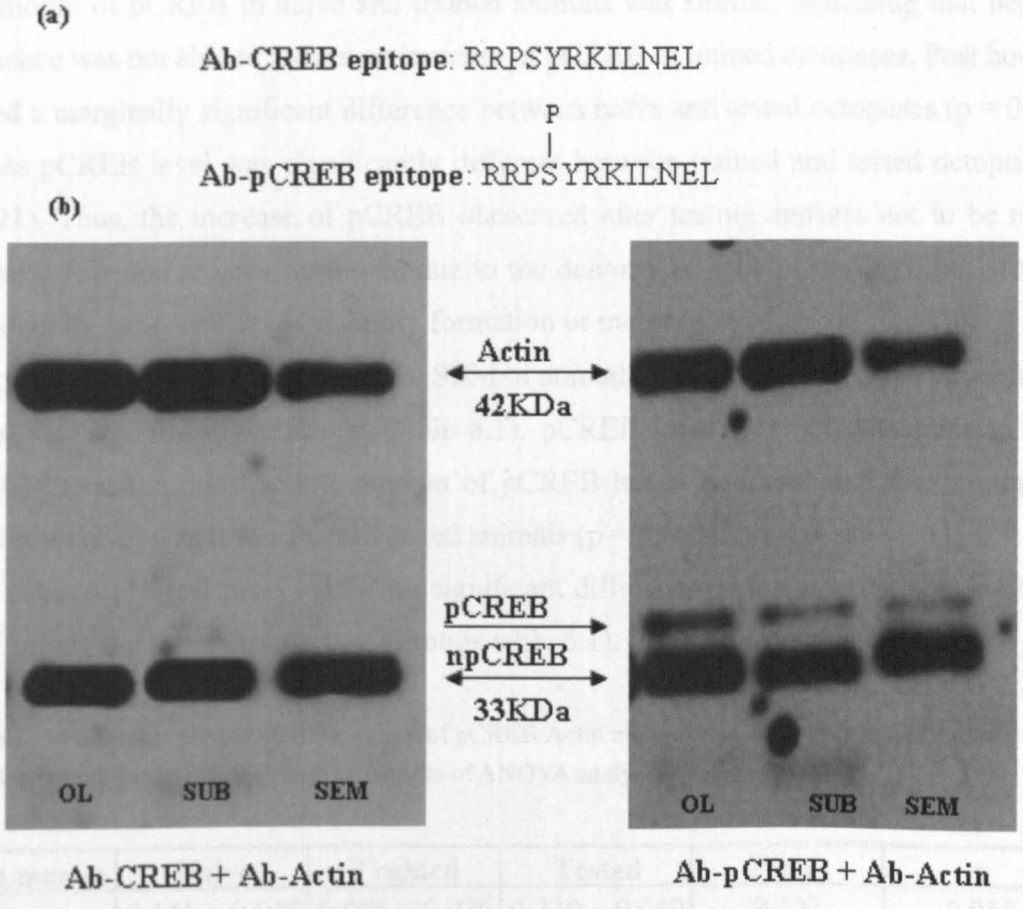


Figure 6.1: Custom octopus antibodies specificity. (a) Octopus epitope sequences used to produce custom antibodies specific for phosphorylated (pCREB) and not-phosphorylated (npCREB) form of CREB. (b) Representative western blot images showing the immunoreactivity and specificity of antibodies (Ab-Actin,OvAb-CREB and OvAb-pCREB).

6.2.2 CREB phosphorylation is induced after fear conditioning

I measured the level of phosphorylated CREB (pCREB) and non phosphorylated CREB (npCREB) in each brain mass of octopuses of three experimental groups.

The amount of npCREB did not change between the groups except in the supraoesophageal mass (SEM; $F_{(2,6)} = 6.733$; $p = 0.029$). Post hoc analysis revealed that the quantity of npCREB were significant by different among naïve and tested octopuses ($p = 0.035$).

For the sake of simplicity I will summarize the results of levels of pCREB after fear conditioning for each brain mass (Fig. 6.2).

The pCREB level in the OL was significantly different among the three experimental groups (table 6.1).

The amount of pCREB in naïve and trained animals was similar, indicating that negative experience was not able to induce an increase of pCREB in trained octopuses. Post hoc tests showed a marginally significant difference between naïve and tested octopuses ($p = 0.052$), whereas pCREB level was significantly different between trained and tested octopuses ($p = 0.021$). Thus, the increase of pCREB obeserved after testing appears not to be related to general fear and arousal responses due to the delivery of shock (training), but probably dependent by a mechanism of memory formation or memory recall.

The amount of pCREB measured in the SEM of animals belonging to the three experimental groups was significantly different (table 6.1). pCREB level was not detectable in naïve animals. Instead, a considerable amount of pCREB has been found in trained octopuses, however its level was lower than in tested animals ($p = 0.001$).

In the suboesophageal mass (SUB) no significant difference in the amount of pCREB was found among the three experimental groups table 6.1).

Table 6.1: In this table are reported the values of pCREB/Actin measured in each brain mass of naïve, trained and tested animal group together with the results of ANOVA analysis.

Brain masses	Naive	Trained	Tested	F(2,6)	p
OL	0.141 ± 0.045	0.096 ± 0.026	0.330 ± 0.049	9.121	0.015
SEM		0.015 ± 0.004	0.098 ± 0.013	46.761	< 0.001
SUB	0.029 ± 0.014	0.021 ± 0.013	0.041 ± 0.019	0.440	0.663

In addition, the Western blots have been conducted also on samples obtained by pooling each brain mass belonging to octopuses of the same experimental group (figure 6.3). The results of these analysis confirmed the previous ones.

The highest quantity of pCREB has been found in OL compared to all other examined masses. In particular, the most abundant level of activated CREB has been revealed in the

OL of tested octopuses. In this case, the pCREB was 3.3 fold more abundant than in the OL of trained octopuses and only 1.7 fold more than naïve. Moreover pCREB was more abundant in the OL of naïve than in the trained (1.4 fold).

In the SEM of the naïve animals has not been found the phosphorylated form of CREB, whereas it was observed in the SEM of trained octopuses. In the tested animals the pCREB level increased 4.5 times.

In the pools of SUB, the lowest amount of activated CREB was found in the trained octopuses, whereas the highest quantity was observed in the octopuses tested after training. The amount of pCREB was not very different among the SUB of the three experimental groups, in fact the pCREB was only 1.6 fold more expressed in the tested octopuses compared to the trained ones, which were the most divergent groups in terms of pCREB quantity.

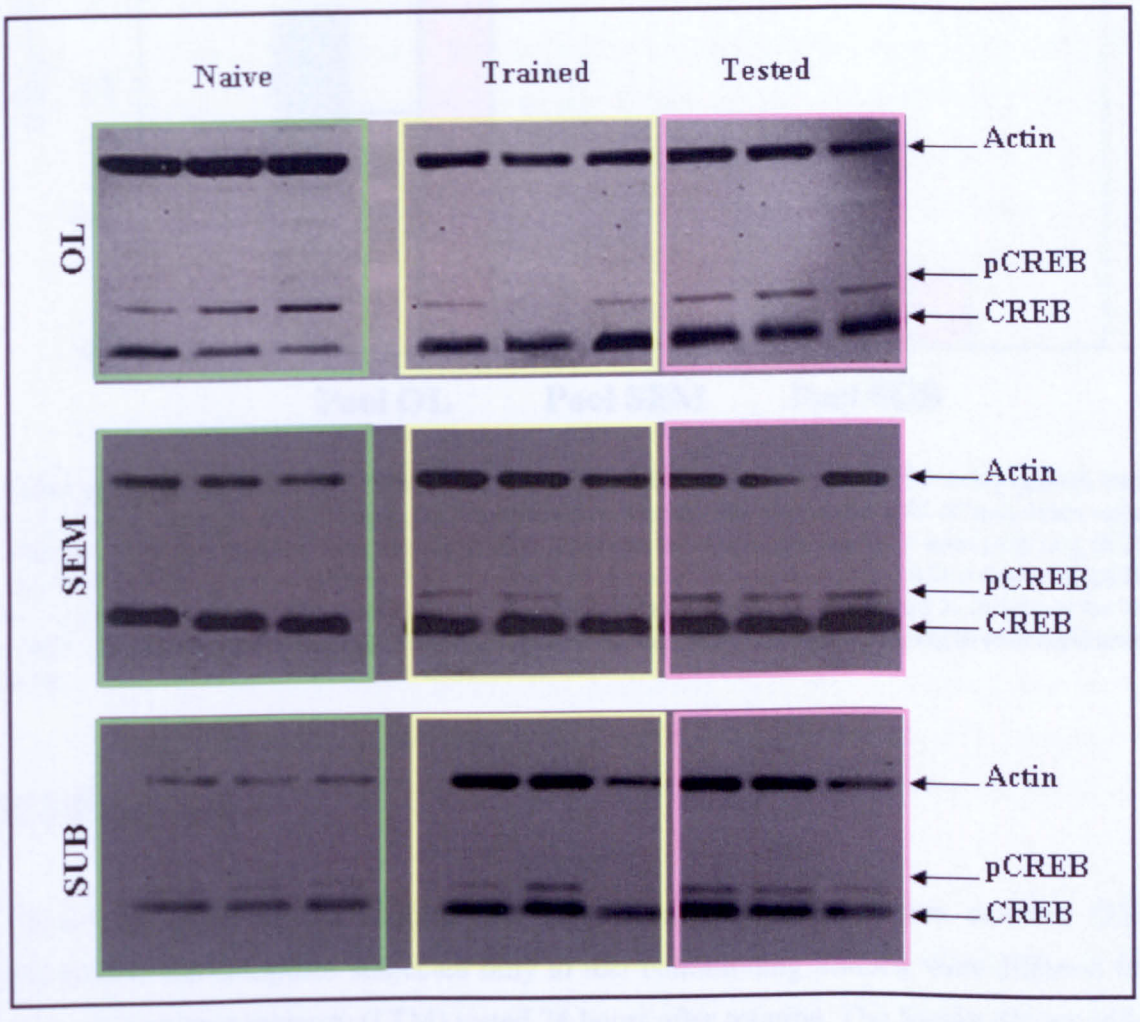


Figure 6.2 Western blots of octopus brain extracts showing actin (42KDa), pCREB and CREB (33KDa), as detected by reaction with custom antibody OvAb-pCREB and commercially available antibody Ab-Actin. Protein levels were normalized to Actin and the pCREB levels have been calculated as ratio between phospho-CREB signal (pCREB) and Actin (pCREB/Actin). ANOVA analysis have been conducted on pCREB/Actin values to compare its amount in each brain mass between the animals of three experimental groups.

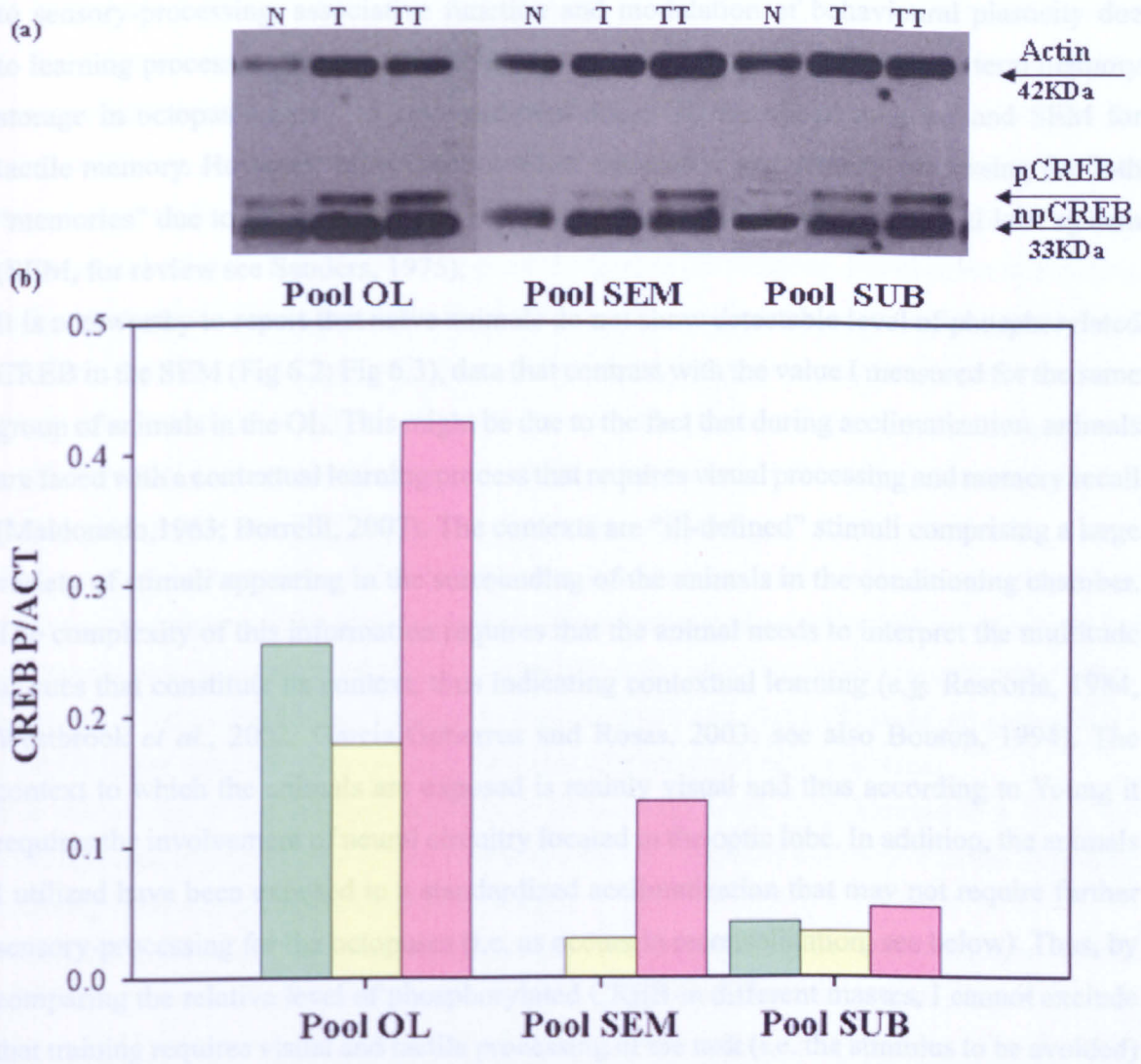


Figure 6.3 Quantitative analysis of protein levels in the pool of each brain mass of the naïve (green), trained (yellow) and tested (pink) octopuses. (a) Representative western blot images for pool of each brain mass of octopuses belonging to three experimental groups. The reported signals are related to actin (42KDa), pCREB and CREB (33KDa), they are detected by reaction with Ab-pCREB and Ab-Actin. (b) The amount of pCREB are reported in the graph, the absolute value of phosphorylated protein is normalized in respect to the actin amount. The normalized amount of pCREB is reported for the pool of masses belonging to each experimental group.

6.3 Discussion

These preliminary results suggest that the mechanisms of short term memory (STM) observed in the octopuses subjected only to fear conditioning training were different from those of long term memory (LTM) tested 24 hours after training. The former did not induce a significant increase of phosphorylated CREB whereas testing after 24 hours showed significantly increased pCREB level compared to the controls.

It is interesting to note that the increase I observed occurs only in SEM and OL and not in the SUB. This seems to be in agreement with the modular organization of the cephalopod brain that considers SUB involved essentially in motor control, while SEM and OL to be devoted

to sensory-processing, associative function and modulation of behavioural plasticity due to learning processes (Young, 1991). According to Young (1991, 1995) long term memory storage in octopus appears to have different sites: OL for visual memory and SEM for tactile memory. However, SEM is involved in associative and sensory-processing for both "memories" due to the amplifier and read-in read-out functions of the vertical lobe system (SEM, for review see Sanders, 1975).

It is noteworthy to report that naïve animals do not show detectable level of phosphorylated CREB in the SEM (Fig 6.2; Fig 6.3), data that contrast with the value I measured for the same group of animals in the OL. This might be due to the fact that during acclimatization, animals are faced with a contextual learning process that requires visual processing and memory recall (Maldonado,1963; Borrelli, 2007). The contexts are "ill-defined" stimuli comprising a large variety of stimuli appearing in the surrounding of the animals in the conditioning chamber. The complexity of this information requires that the animal needs to interpret the multitude of cues that constitute its context, thus indicating contextual learning (*e.g.* Rescorla, 1984; Westbrook *et al.*, 2002; Garcia-Gutierrez and Rosas, 2003; see also Bouton, 1994). The context to which the animals are exposed is mainly visual and thus according to Young it requires the involvement of neural circuitry located in the optic lobe. In addition, the animals I utilized have been exposed to a standardized acclimatization that may not require further sensory-processing for the octopuses (i.e. as occurs in reconsolidation, see below). Thus, by comparing the relative level of phosphorylated CREB in different masses, I cannot exclude that training requires visual and tactile processing of the task (i.e. the stimulus to be avoided) that is not sufficient to increase CREB activity, as it seems to occur in my experiments one hour after testing.

In addition, considering the fact that the brain of octopuses seems to have a modular organization, we may expect that the levels of phosphorylation I detected in different masses are different in amplitude considering the significant difference in the number of cells involved in LTM storage and processing for visual (i.e. OL) and for tactile memories (i.e. inferior frontal lobe system in the SEM) that appears to be 60 fold more abundant in the system for visual memory (see Appendix; Young, 1964; 1991).

A growing amount of evidence in both vertebrates and invertebrates documents CREB activation as an essential step in the processes of learning and long-term memory formation. The preliminary results of this study seem to suggest that the processes of learning and memory in octopuses, activated after fear conditioning training, could be related to molecular mechanisms known to be involved in other organisms such as *A. californica* (Kandel, 2001) or *M. musculus* (Impey *et al.*, 1998). CREB phosphorylation appears to be an essential event for long-term fear conditioning memory and for the retrieval of long-term memory once it has been formed in octopus.

It is important to note that in vertebrates the re-exposure to the conditioned stimulus (CS) presented during the fear conditioning training triggers two different processes: reconsolidation and extinction memory. In particular brief re-exposure to the CS activates the reconsolidation process that serves to stabilize or maintain the original memory of training experience, while more prolonged re-exposure leads to the induction of extinction of fear. Anyway in both processes CREB and gene expression are required, but they are activated in different regions of brain on the basis of the kind of re-exposure (brief or prolonged). After brief re-exposure, significant activation of CREB-mediated gene expression is observed in the hippocampus and amygdala. In contrast, after the prolonged re-exposure, a significant activation of CREB-mediated gene expression is registered in the amygdala and prefrontal cortex (Mamiya *et al.*, 2009; Radulovic & Tronson, 2010).

The repeated presentation of the stimulus (5 trials, tesing phase) should be considered as a prolonged exposure to CS, even in absence of the delivery of negative reinforcement carried out twenty-four hours after training. This might induce extinction rather than reconsolidation. However I found that during testing savings occurred and that I observed an increased of pCREB in the optic lobes suggesting the onset of a reconsolidation process.

It is noteworthy to remind that, similarly to what occurs in the hippocampus (e.g. Bliss and Collingridge, 1993; Alberini *et al.*, 1999), the OL of octopuses play a role in the formation and long-term storage of memory processes.

This could then justify the activation of a reconsolidation process that requires phosphorylation of CREB in two areas of the octopus brain namely optic lobes and supraoesophageal mass as occurs in hippocampus and amygdale of vertebrates. It would be interesting to analyze the variation of the activation of CREB after a single trial of the test and after a longer exposure to the CS.

CHAPTER 7

FEAR CONDITIONING IN *OCTOPUS VULGARIS*: MOLECULAR ANALYSIS OF THE CIRCUITRY

7.1 Analysis of gene expression in response to fear conditioning

To study the relationship between the learning processes and gene expression in octopus, I carried out real-time quantitative PCR experiments on samples taken from naïve and fear-conditioned octopuses.

Since the brain of the octopus is organized into lobes that serve different regulatory functions (i.e. visual and tactile systems do not share all structures involved in the processing, see Young, 1991; Borrelli and Fiorito, 2008), I have studied the variation of the expression of target genes in response to behavioural experience in the brain masses (SEM, SUB and OLs; paragraph 7.3) and, again, in sub-sectors of each mass. For this reason it was adopted a strategy that allowed me to observe the change of expression of genes in some sub-regions of each mass which roughly represented each lobe (paragraph 7.4).

As reported in the previous sections of this thesis, I used a biased approach to select genes to be used as targets (Ubiquitin hydrolase, stathmin, tyrosine hydroxylase and dopamine transporter) also on the basis of the available information on their involvement in reward and learning and memory processes.

In particular, ubiquitin hydrolase has been shown to be required for maintenance of memory in the passive avoidance tests; the lack of its expression is also known to impair memory and LTP formation (Sakurai *et al.*, 2008); stathmin is required for the expression of innate fear and for the formation of memory after fear conditioning, as shown in stathmin knockout mice (e.g. Shumyatsky *et al.*, 2005). In addition, tyrosine hydroxylase (TH) and dopamine transporter (dat) are involved in dopaminergic modulation. TH is the rate-limiting enzyme for dopamine and noradrenaline biosynthesis (e.g. Jones *et al.*, 1998, Jaber *et al.*, 1999). Dat clears dopamine from the extra-cellular space and serves as important regulator of signal amplitude and duration at dopaminergic synapses (Mortensen & Amara, 2003). Both TH and dat are considered important for recall of fear conditioning (Zhang *et al.*, 2008; Kobayashi

et al., 2000; Kobayashi and Kobayashi, 2001).

7.2 Materials and methods

7.2.1 Subjects

A total of 12 *Octopus vulgaris* of both sexes from 200 to 400 g were caught in the Bay of Napoli (Italy) during the summer of 2007. The octopuses were randomly assigned to control (sacrificed one hour after their arrival in laboratory; Naive, N = 6) and experimental group (sacrificed one hour after test session of fear conditioning TT, N = 6). Training protocols for fear conditioning are reported above (see chapter 2).

7.2.2 Samples-Fear conditioning

After dissection, the different parts' of the brain (OL, SEM, SUB) were placed in plastic moulds (Peel – A – Way Disposable Embedding Molds 22 x 22 mm Polyscience Inc.- Warrington PA USA), immediately frozen in liquid nitrogen and stored at -80°C until processed.

Each brain mass was fixed onto a sample holder using a small quantity of Tissue-Tech O.C.T. (optimum cutting temperature) embedding compound. Brain parts were placed on the holder by their foremost posterior part and sectioned by the cryostat Leica CM3050 S (Leica, Milano, Italy) following the anterior-posterior axis. Sections (20 µm thick) were collected over SuperFrost Plus Microscope Slides (VWR International, Milano, Italy) marked with five different colours.

For every brain mass, a set of five slide series were prepared together with 1 series of Eppendorf tubes as illustrated in figure 7.1.

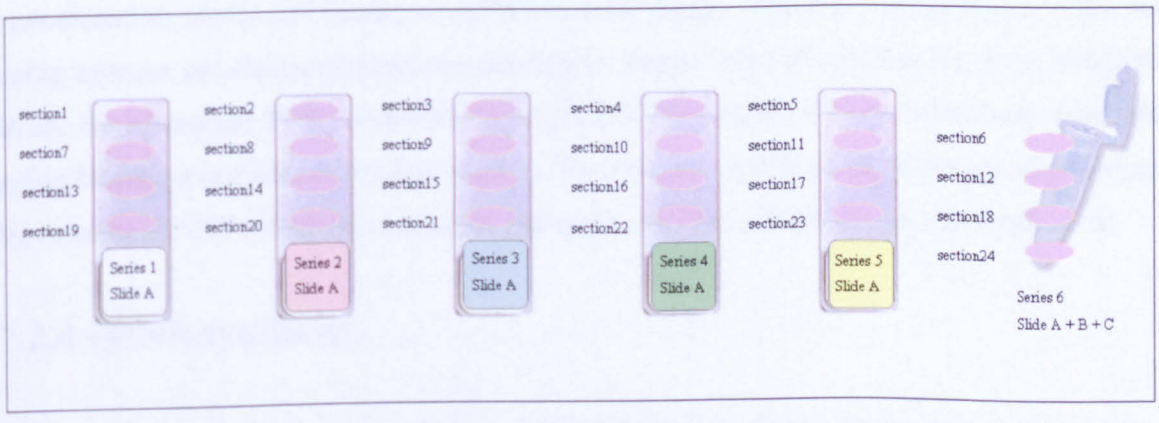


Figure 7.1 : Preparation of octopus brain section series. Coronal brain sections from each part of CNS were collected constructing six series: 5 slide series and 1 tube series.

On each slide, 4 slices were collected, whereas in each tube twelve slices were retained (minimal number of slices necessary to extract almost 500 ng of RNA).
The brain slices collected on the slides were stored at -80°C until used. Slices collected in tubes for RNA analysis were supplemented with buffer Eurozol (EuroClone, Pavia, Italy) before storing at -80°C. The slices of SEM and SUB were supplemented with 800 µl of Eurozol, whereas OL slices had 1 ml of buffer.

7.2.3 RNA isolation and quantification

Filtered tips, DEPC-treated sterile water, and autoclaved microcentrifuge tubes were used for all subsequent steps. Slices of tissue were homogenized in Eurozol (EuroClone, Pavia, Italy) simply by vortexing the samples and incubated at room temperature for 5 minutes. Chloroform was added in a proportion of 1:10, mixed carefully and incubated on ice for 5 minutes. Samples were centrifuged at 12,000 g for 30 min at 4°C. The extraction with chloroform was repeated twice. The aqueous phase was transferred into new tubes, where one volume of cold isopropanol and 1 µl of glycogen were added. After gentle mixing, samples were incubated overnight at -20°C. Centrifugation for 30 minutes at 4°C and at 12,000 g was followed by aspiration of the supernatant. Pellets were washed three times with 1 ml of 75% ethanol, and then centrifuged for 15 minutes at 4°C at full speed. The supernatant was aspirated and samples were dried in the Vacufuge concentrator model 5301 (Eppendorf, Hamburg, Germany) for 10 minutes. After resuspension in DEPC treated water, RNA samples were treated with TURBO DNase (Ambion, Austin, TX) to remove any contaminating genomic DNA. The samples were incubated at 37°C for 30 minutes, then 0.1 volume of DNase inactivation reagent was added and the RNA was transferred to a new tube after a step of centrifugation at 10000 g for 1.5 minutes. RNA optical density measurements at 230, 260 and 280 nm were read using the Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies). This spectrophotometer was used to assess the concentration, purity and quality of mRNA in each sample. The RNA integrity was evaluated using agarose gel electrophoresis visualizing the fragments representing the most abundant RNA: the ribosomal RNA. Intact bands of 28S and 18S rRNA subunits were observed on the gel indicating a minimal RNA degradation. The absence of DNA contamination was verified by running a PCR with β-actin primers and analyzing the sample by gel electrophoresis.

7.2.4 cDNA synthesis

cDNA was synthesized using 0.5 µg of total RNA per sample. The master mix consisted of 0.5 mM dNTP, 25 ng/µl oligo dT, 2.5 ng/µl random hexamers, 1 x RT reaction buffer,

5mM $MgCl_2$, 0.01M DTT, RNaseOUT Recombinant Ribonuclease Inhibitor and 2.5 U/µl of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Samples were incubated for 10 minutes at 25°C and 50 minutes at 42°C. The reaction was terminated with a cycle at 70 °C for 15 minutes followed by a treatment with RNase H at 37°C for 20 minutes. Prior to use in RT qPCR experiments, cDNA was diluted 1:20 with sterile H_2O and it was stored at -20°C until further use.

7.2.5 Real-time qPCR

Two µl of diluted cDNA were used in a SYBR Green PCR for each reaction. Polymerase chain reactions were carried out in an optical 384-well plate with an ABI PRISM1 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA), using FastStart SYBR Green Master mix (Roche, Indianapolis, IN) to monitor dsDNA synthesis. Reactions (total volume: 5 µl) contained: 2 µl cDNA, 2.5 µl SYBR Green Master mix reagent, 0.3 µM (each) of forward and reverse primers. The following thermal profile was used: 95°C for 10 min, one cycle for cDNA denaturation; 95°C for 15 sec and 60°C for 1 min, 40 cycles for amplification; 72°C for 5 min, one cycle for final elongation; one cycle for melting curve analysis, from 60°C to 95°C to verify the presence of a single product. PCR data were analyzed using the SDS 2.2.2 software (Applied Biosystems) to determine cycle threshold (Ct) values. Each assay included a no-template control for every primer pair and a standard curve with 1:10, 1:25, 1:50, 1:100, 1:200 dilutions of the standard cDNA. The standard sample was a pool of equal amount of 6 randomly chosen samples belonging to the three octopus brain masses. To capture inter-assay variability all RT qPCR plates contained inter-run calibrators.

7.2.6 Primer design: efficiency and specificity

Primers were designed by Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) using sequences for specific octopus mRNA. Primer parameters were set to 20 nucleotides in length, product size 100-150 base pairs and melting point 58 - 60°C. Sequences of primers utilized for RT qPCR experiments are listed together with amplicon size and efficiency in the table 7.1.

Target sequences amplified by the primer pairs were evaluated with the MFOLD software (http://www.bioinfo.rpi.edu/applications/mfold/) in order to check for the formation of secondary structures at the site of primer binding.

The efficiency evaluation is an essential step for the real-time gene quantification procedure. The assessment of the exact amplification efficiency of target and reference genes must

be carried out before any calculation of relative gene expression. The efficiency of each pair of primers has been calculated according to Standard method curves with the equation $E = 10^{-1/slope}$ (Pfaffl *et al.*, 2002; Radonic *et al.*, 2004). Five serial dilutions (1:10, 1:25, 1:50, 1:100, 1:200) of a standard sample were made to determine the efficiency of reactions conducted with each pair of primers. Standard curves were generated for each sample/gene combinations using the Ct value versus the logarithm of each dilution factor. Efficiency values were taken into account in all subsequent calculation.

The melting curve of each sample was analyzed to confirm the specificity of the primers and to be sure of the nature of PCR products.

PCR products were analyzed by agarose gel electrophoresis to confirm the presence of a single band.

Table 7.1 : Primer sequences (F:forward; R:reverse), amplicon size and amplification efficiency (E) of reference and target genes.

Gene	GenBank acc. num.	Gene Ontology*		Primer sequence 5' - 3'	Amplicon size (bp)	E
Ov – dat	FJ617441	Dopamine transmembrane transporter activity (Fu) GO: 0005329	F	GCCCTAGACGGCATCAAATA	109	2
			R	ATCCTGGTCCAAGGGAAAAG		
Ov – stmn	GQ152874	Micritubule disassembly (P) GO: 0007019	F	TGGAGAGAAAAGGCCAAAGA	133	2
			R	CAATAGCCTCCTGGGTGAGA		
Ov – tuba	X15845	Microtubule (C) GO: 0005874	F	ACTGGTGTCCAACTGGCTTC	105	2
			R	TGCTTAACATGCACACAGCA		
Ov – TH	FJ617442	Peptidyl-tyrosine hydroxilation (P) GO: 0018336	F	CTCATTGCAGACATGGCATT	128	2
			R	GCGTGAGTCGGAAACAGATT		
Ov - ub/S27A	FJ617440	Protein ubiquitination (P) GO: 0016567	F	TCAAAACCGCCAACTTAACC	113	2
			R	CCTTCATTTGGTCCTTCGTC		
Ov – uch	GQ148556	Deubiquitination (P) GO: 0006514	F	CGATTGTTTGACGGATGAAA	112	2
			R	AAAAAGCCTTTAGCTGTGATGTT		

* The biological function (Fu), biological process (P), cellular components (C) accompanied by a GO number has been listed for each gene according to http://www.geneontology.org/

7.2.7 Reference genes and normalization

As internal control genes I uitlized reference genes already selected for *O. vulgaris* (*Ov-tubA* and *Ov-ubi/S27A;* Sirakov *et al.*, 2009).

The gene stability analysis was conducted using the geNorm software (http://medgen.ugent.be/_jvdesomp/genorm/).

The M value was calculated using geNorm software. This value was lower than 1.5 when the pairwise variation was analyzed for each mass singularly such as when it was calculated for all masses together, indicating that these genes were steadily expressed in the central nervous

system of both control and trained octopuses. For the study of stability of candidate reference genes see Appendix 3. The normalization factor was calculated using the geometric mean of reference genes, and utilized to quantity was used to calculate the relative expression level of target genes.

7.3 Results: target gene expression in the masses of *O. vulgaris* CNS

Quantitative real-time PCR was used to study the expression of four genes of interest in the octopus brain masses (SEM, SUB, OL) in response to fear conditioning.

In order to identify the transcriptional regulation of target genes induced by behavioural experiences, the expression level of each gene has been studied in the brain of six control and six trained octopuses. The brains have been dissected and the RNA of each brain mass was analyzed as described in materials and methods (paragraph 7.2.3)

A multivariate test was conducted to consider the relationship between variation of gene expression (dependent variables) and other factors (independent variables): brain mass and behavioural experience. The target genes were differently expressed in the octopus brain masses and they are regulated in different way in response to the behavioural experiences as reported in table 7.2.

Table 7.2: After MANOVA variation of target gene expression in response to the independent variables (behavioural experience and mass).

Multivariate test

Independent variable	*df*	*F*	*P*
Intercept	4, 57	69.088	**< 0.0001**
Behavioural experience	4, 57	6.757	**< 0.0001**
Mass	8, 116	20.298	**< 0.0001**
Behavioural experience x Mass	8, 116	6.259	**< 0.0001**

In addiction I controlled any difference in the gene expression due to sex (table 7.3).

Table 7.3: After MANOVA variation of target gene expression in response to the independent variables (sex and mass).

Multivariate test

Independent variable	*df*	*F*	*P*
Intercept	4, 57	40.724	**< 0.0001**
Mass	8, 116	12.931	**< 0.0001**
Sex	4, 57	1.451	0.229
Mass x Sex	8, 116	0.292	0.967

Since gene expression was different between different brain masses, the aim of successive MANOVA analysis has been to evaluate if the target gene expression could differentially change in each mass in response to behavioural experience (table 7.4) I found that in the OL the amount of mRNA of target genes changed in response to fear conditioning. Similar results can be observed for the SUB, whereas no significant differences have been measured in the SEM between naïve and trained octopuses.

Table 7.4: After MANOVA variation of target gene expression in each mass in response to behavioural experience.

Multivariate test

	OL			*SEM*			*SUB*		
Independent variable	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Intercept	4, 17	34.234	**< 0.0001**	4, 17	23.589	**< 0.0001**	4, 17	20.620	**< 0.0001**
Behavioural experience	4, 17	12.622	**< 0.0001**	4, 17	1.581	0.2250	4, 17	3.560	**0.0280**

In the following pages, I have analyzed the expression level of four target genes (ubiquitin hydrolase, stathmin, dopamine transporter, Tyrosine Hydroxylase) within the different masses central nervous system of *O. vulgaris* to evaluate their putative involvement in response to fear conditioning.

For sake of clarity, the results of expression level of each target gene are discussed separately.

7.3.1 Ubiquitin hydrolase

O. vulgaris ubiquitin C-terminal hydrolase (*Ov-uch*) expression data are summarized in figure 7.2. Analyzing the *Ov-uch* expression in OL, in SEM and in SUB it seemed different between the masses in each experimental group and this observation was confirmed by ANOVA analysis and post hoc Bonferroni test ($F_{(2, 27)} = 7.405$, $p = 0.003$ for naïve group; $F_{(2, 33)} = 4.722$, $p = 0.016$ for fear conditioning group). In particular, in both experimental groups *Ov-uch* is expressed more in SUB than in OL and SEM, even if the significant differences were present between SUB and OL (OL vs SEM $p = 0.338$, OL vs SUB $p = 0.002$, SEM vs SUB $p = 0.111$ for naïve group; OL vs SEM $p = 0.013$, OL vs SUB $p = 0.013$, SEM vs SUB $p = 0.227$, for fear conditioning group).

In order to analyze the effect of fear conditioning on *Ov-uch* expression, ANOVA analysis was conducted. The target gene expression in every brain mass was not significantly changed by behavioural experience ($F_{(1, 20)} = 1.280$, $p = 0.271$ for OL; $F_{(1, 20)} = 2.450$, $p = 0.133$ for SEM; $F_{(1, 20)} = 1.802$, $p = 0.195$ for SUB). These results suggested that *Ov-uch* is not transcriptionally regulated in response to fear conditioning and probably is not involved in the molecular mechanisms regulating consolidation of learned fear.

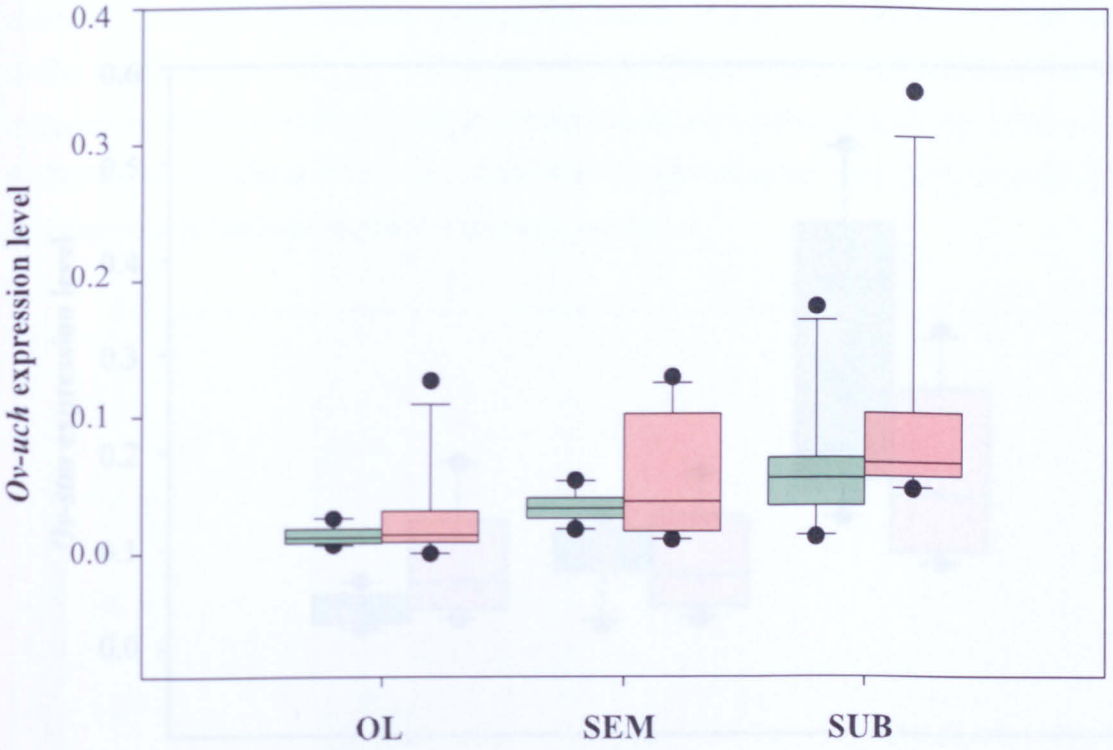


Figure 7.2 : *ubiquitin C-terminal hydrolase* expression level in OL, SEM and SUB. The *Ov-uch* quantity has been normalized using two reference genes: *Ov-ubi* and *Ov-tubA* as reported in materials and methods. The distribution is shown in green (naïve group) and pink (fear conditioning group) vertical box plot as median (lines), 25th and 75th percentiles (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers.

7.3.2 Stathmin

O. vulgaris stathmin (*Ov-stm*) expression data are summarized in the figure 7.3. *Ov-stm* was differently expressed in the brain masses of each experimental group using ANOVA analysis and Bonferroni post hoc test ($F_{(2, 27)} = 19.965$, $p < 0.0001$ for naïve group; $F_{(2, 33)} = 6.461$, $p = 0.004$ for fear conditioning group). In particular, in both experimental groups significant differences were found between *Ov-stm* expression in SUB compared to all other masses (OL vs SEM $p = 0.650$, OL vs SUB $p < 0.0001$, SEM vs SUB $p < 0.0001$ for naïve group; OL vs SEM $p = 1$, OL vs SUB $p = 0.010$, SEM vs SUB $p = 0.013$ for fear conditioning group). Fear conditioning induced changes in OL *Ov-stm* expression, because the target gene was significantly more expressed (2 folds) in the OL of octopuses trained compared to naïve ($F_{(1, 20)} = 5.454$, $p = 0.030$).

In SUB a marginally significant decrease of *Ov-stm* expression level (0.62 folds) was revealed in response to fear conditioning, confirming that the behavioural experience seemed to induce changes in the *Ov-stm* mRNA availability ($F_{(1, 20)} = 4.369$, $p = 0.050$).

In contrast no significant differences emerged when the *Ov-stm* expression level was compared between SEMs of the two experimental groups ($F_{(1, 20)} = 0.032$, $p = 0.860$).

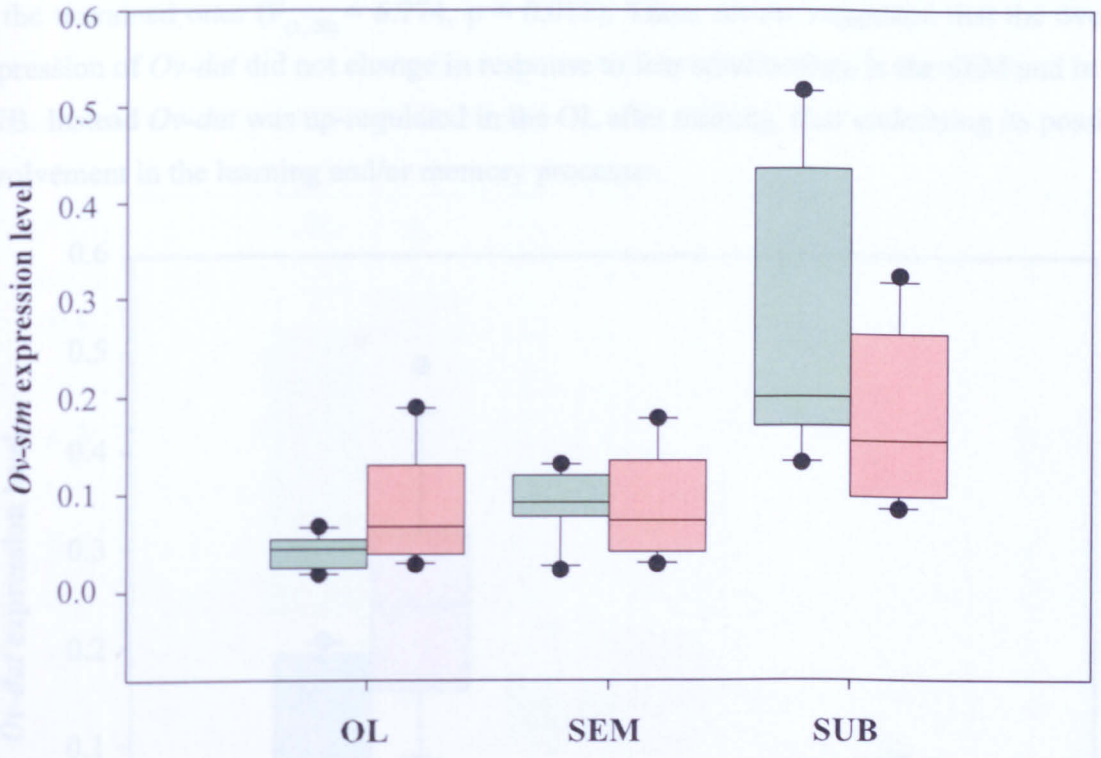


Figure 7.3 : *stathmin* expression level in OL, SEM and SUB. Stathmin quantity has been normalized using normalization factor generated by GeNorm with two reference genes *Ov-tubA* and *Ov-ubi* as reported in Materials and methods. The distribution is shown by green (naïve group) and pink (fear conditioning group) vertical box plot as median (lines), 25th and 75th percentiles (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

7.3.3 Dopamine transporter

O. vulgaris dopamine transporter (*Ov-dat*) expression data are summarized in figure 7.4. *Ov-dat* was not equally expressed in the octopus CNS, but its expression level showed significant differences between the brain masses of each experimental group as suggested by ANOVA analysis and Bonferroni post hoc test ($F_{(2, 27)} = 49.911$, $p < 0.0001$ for naïve group; $F_{(2, 33)} = 49.023$, $p < 0.0001$ for fear conditioning group). In particular *Ov-dat* expression in the OL was significantly higher respect to the other masses in both naïve (OL vs SEM $p < 0.0001$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 0.605$) and trained octopuses (OL vs SEM $p < 0.0001$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 1$).

In order to analyze if the behavioural experience was the factor that influenced *Ov-dat* expression in each brain mass an ANOVA analysis has been conducted. The expression level of *Ov-dat* was considered separately for each brain mass. No significant differences of *Ov-dat* mRNA amount were found in SEM ($F_{(1, 20)} = 0.289$, $p = 0.597$) and SUB ($F_{(1, 20)} = 0.942$, $p = 0.343$) comparing the experimental groups (naive and fear conditioning). In contrast, *Ov-*

dat mRNA amount significantly increased in the OL of trained octopuses (1.7 folds) respect to the untrained ones ($F_{(1, 20)} = 6.774$, $p = 0.017$). These results suggested that the overall expression of *Ov-dat* did not change in response to fear conditioning in the SEM and in the SUB. Instead *Ov-dat* was up-regulated in the OL after training, thus underlying its possible involvement in the learning and/or memory processes.

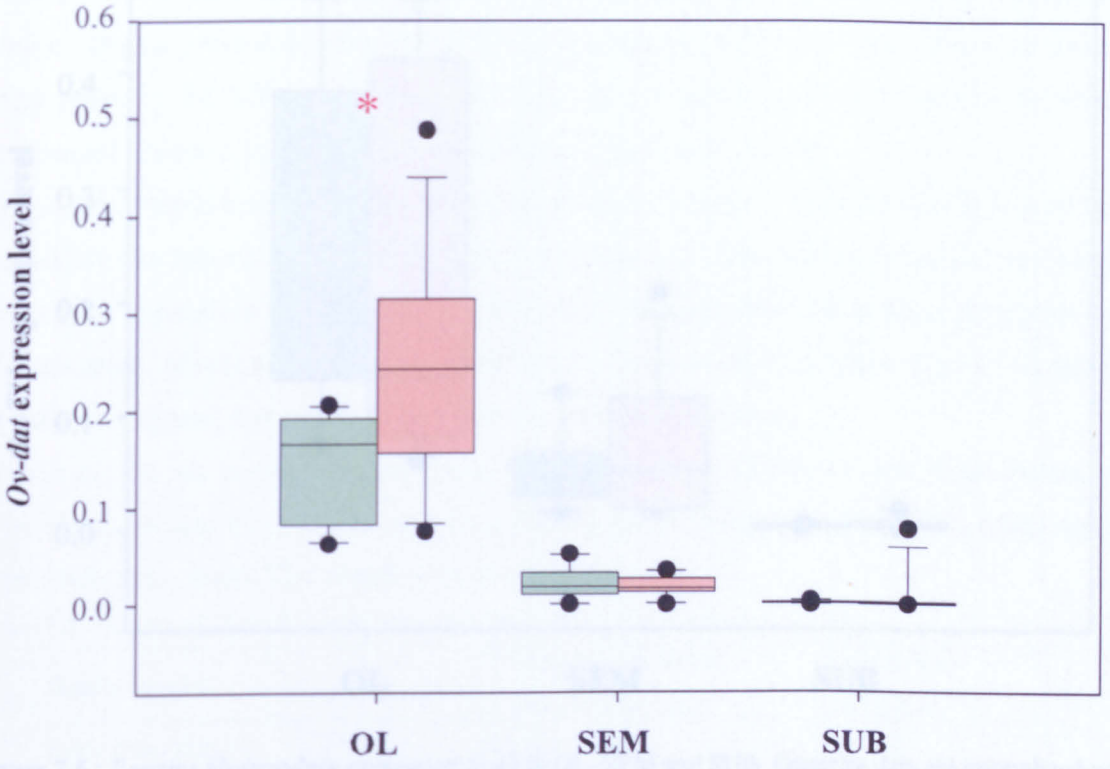


Figure 7.4: *dopamine transporter* expression level in OL, SEM and SUB. *Dat* quantity is normalized using normalization factor generated by GeNorm with two reference genes *Ov-tubA* and *Ov-ubi* as reported in materials and methods. The distribution is shown by green (naïve group) and pink (fear conditioning group) vertical box plot as median (lines), 25th and 75th percentiles (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

7.3.4 Tyrosine hydroxylase

The figure 7.5 summarized the *O. vulgaris* tyrosine hydroxylase (*Ov-TH*) expression data. *Ov-TH* resulted differently expressed in the brain masses of naïve ($F_{(2, 27)} = 23.278$, $p < 0.0001$) and trained octopuses ($F_{(2, 33)} = 13.318$, $p < 0.0001$). The expression levels of *Ov-TH* were significantly higher in OL in comparison with other brain masses in both untrained (OL vs SEM $p < 0.0001$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 0.650$) and trained octopuses (OL vs SEM $p = 0.005$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 0.365$). In order to analyze the effect of fear conditioning training on *Ov-TH* expression an ANOVA analysis has been conducted comparing *Ov-TH* expression level of each mass between the two experimental

groups. Fear conditioning did not induce significant changes of the *Ov-TH* expression neither in the OL ($F_{(1, 20)} = 0.158$, $p = 0.695$), or in SEM ($F_{(1, 20)} = 0.963$, $p = 0.338$) and in SUB ($F_{(1, 20)} = 2.813$, $p = 0.109$). Thus, these results suggested that overall *Ov-TH* expression was not changed in response to fear conditioning in octopus CNS.

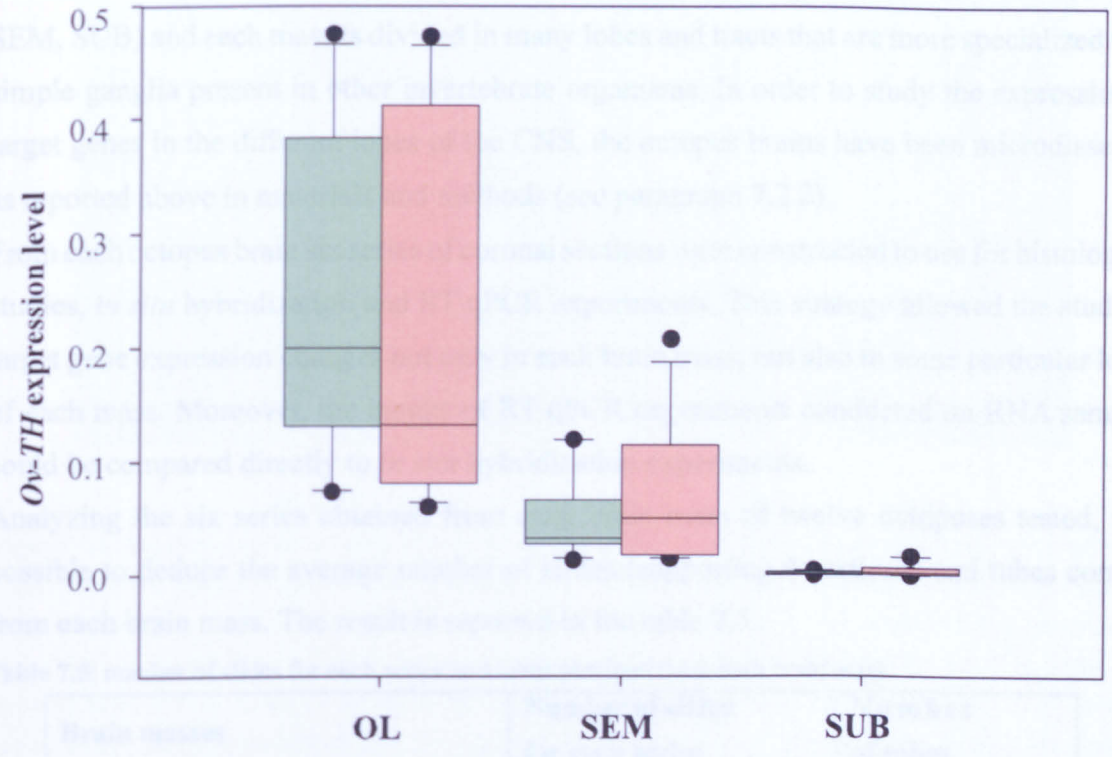


Figure 7.5 : *Tyrosine Hydroxylase* expression level in OL, SEM and SUB. Quantity data are normalized using normalization factor generated by GeNorm using two reference genes *Ov-tubA* and *Ov-ubi* as described in Materials and methods. The distribution is shown by green (naïve group) and pink (fear conditioning group) vertical box plot as median (lines), 25th and 75th percentiles (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers.

7.4 Results: target gene expression in the lobes of *O. vulgaris* brain masses

The octopus has one of the most complex nervous system of all invertebrates. It was estimated to have 300 million neurons. These cells are arranged in different masses (OL, SEM, SUB) and each mass is divided in many lobes and tracts that are more specialized than simple ganglia present in other invertebrate organisms. In order to study the expression of target genes in the different lobes of the CNS, the octopus brains have been microdissected as reported above in materials and methods (see paragraph 7.2.2).

From each octopus brain six series of coronal sections were constructed to use for histological studies, *in situ* hybridization and RT qPCR experiments. This strategy allowed the study of target gene expression changes not only in each brain mass, but also in some particular lobes of each mass. Moreover, the results of RT qPCR experiments conducted on RNA samples could be compared directly to *in situ* hybridization experiments.

Analyzing the six series obtained from each brain mass of twelve octopuses tested, it is possible to deduce the average number of slides (supporting 4 sections) and tubes coming from each brain mass. The result is reported in the table 7.5.

Table 7.5: number of slides for each series and tubes obtained from each brain mass.

Brain masses	**Number of slides for each series**	**Number of tubes**
Optic lobes (OL)	18	6
Supraoesophageal mass (SEM)	12	4
Suboesophageal mass (SUB)	16	5

Thus, SEM sections were divided in four tubes for RT qPCR experiments. By analyzing the slide series dedicated to histological studies it is possible to identify the SEM lobes included in each Eppendorf tube. SEM was subdivided in four regions: SEM c, SEM f, SEM i and SEM l. All regions are reported in figure 8.6 where it is possible to deduce that SEM c region included buccal lobe, inferior frontal lobe, sub-frontal lobe and part of superior frontal lobe. Instead, SEM f was formed by superior frontal lobe, sub-frontal lobe, anterior basal lobe, vertical lobe and sub-vertical lobe. SEM i was composed by anterior and median basal lobe, sub-vertical lobe, vertical lobe and optic commissure. The region SEM l contained median and dorsal basal lobe, sub-vertical lobe and the posterior part of vertical lobe.

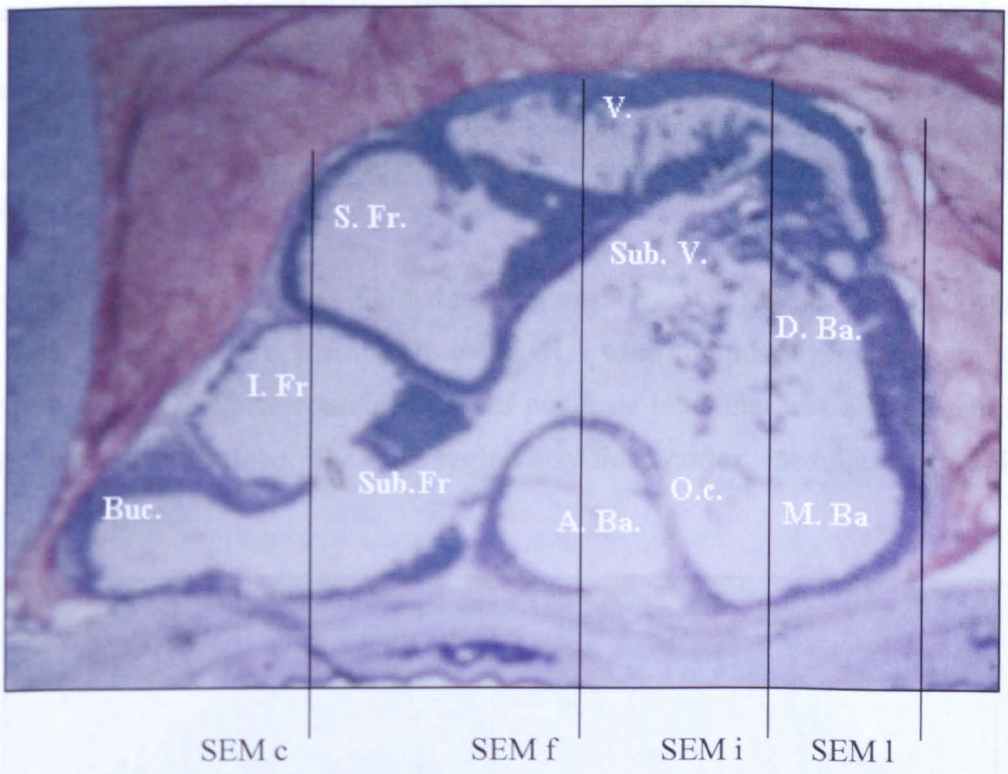


Figure 7.6: Sagittal slice of *O. vulgaris* supra-oesophageal mass (SEM) is represented with its constituent lobes. The brain section is stained with cresyl violet, a Nissl stain that colours cell bodies a brilliant violet. As reported above, the octopus brains are coronally sectioned in six series (five slice series collected on slides and one series into tubes). The vertical lines define the brain areas contained in each tube of sixth SEM series. This series of SEM coronal slices are composed of four tubes named regions SEM c, SEM f, SEM i, SEM l. (Bucc.: buccal lobe, I. Fr.: inferior frontal lobe, Sub. Fr.: sub-frontal lobe, S. Fr.: superior frontal lobe, A. Ba.: anterior basal lobe, V.: vertical lobe, Sub. V.: sub-vertical lobe, O. c.: optic commissure, D. Ba: dorsal basal lobe, M. Ba.: median basal lobe; see Appendix 3 for details on sections).

As reported in table 8.5, SUB was divided in 5 regions (SUB c, SUB f, SUB i, SUB l, SUB o; Figure 7.7).

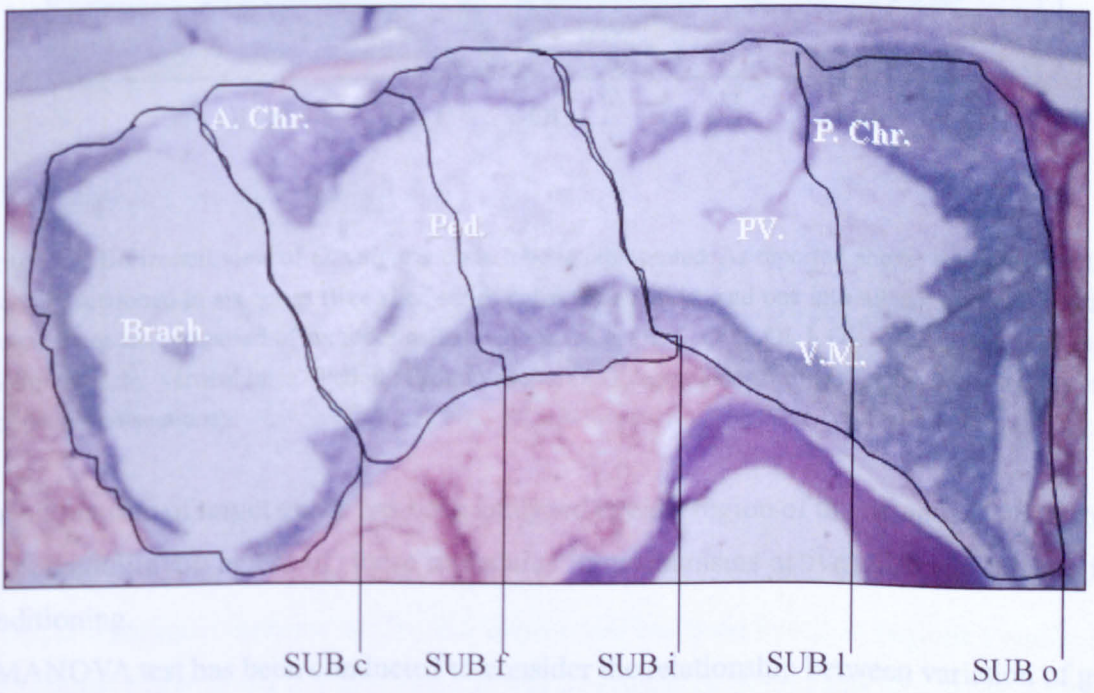


Figure 7.7: Sagittal slice of *O. vulgaris* sub-oesophageal mass is represented with its constituent lobes. The

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brain section is stained with cresyl violet, a Nissl stain that colors cell bodies a brilliant violet. As reported above, the octopus brains are coronally sectioned in six series (five slice series collected on slides and one into tubes). Sixth series of SUB coronal slices are composed of five tubes named regions SUB c, SUB f, SUB i, SUB l, SUB o. (Brach.: brachial lobe, A.Chr.: anterior chromatophore lobe, Ped.: Pedal lobe, P. V.: Palliovisceral lobe, V. M.: Vasomotor lobe, P. Chr. : Posterior chromatophore lobe; see Appendix 3 for details on sections).

As reported in table 7.5 , OL was divided in 6 regions: OL c, OL f, OL i, OL l, OL o, OLr (see figure 7.8).The region OL c, just the OL o and OL r were composed principally by medulla and cortex, while OL f also contained part of peduncle lobe, that was also present in OL i together with olfactory lobe. The OL l region contained cortex, medulla, part of olfactory lobe and optic gland.

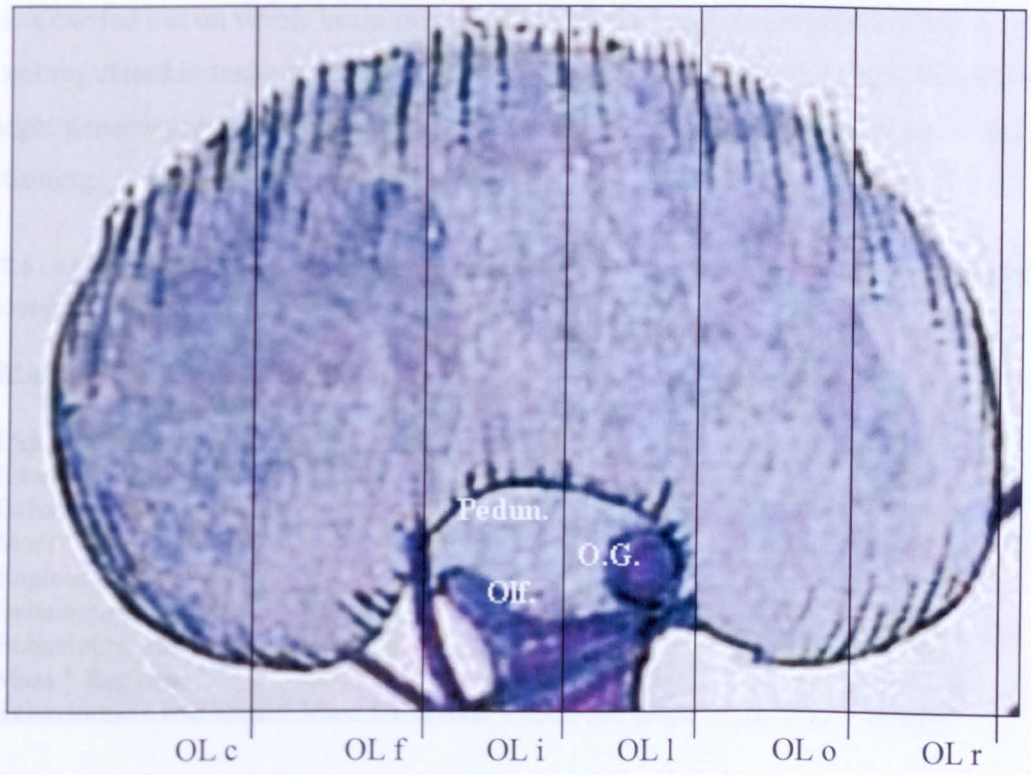


Figure 7.8: Horizontal view of *O. vulgaris* optic lobe is represented. As reported above, the octopus OL are coronally sectioned in six series (five slice series collected on slides and one into tubes). Sixth series of OL coronal slices are composed of six tubes called regions OL c, OL f, OL i, OL l, OL o, OL r . Each OL region is delimitated by vertical lines. (Pedun.: Peduncle lobe, O. G.: optic gland, Olf.: Olfactory lobe; see Appendix 3 for details on sections).

The expression of target genes has been analyzed in each region of the octopus CNS to study the potential involvement of these molecules in mechanisms activated in response to fear conditioning.

A MANOVA test has been conducted to consider the relationship between variation of gene expression in response to different behavioural experience and all independent variables that

could potentially influence the regulation of genes of interest: brain mass and brain regions (table 7.6). The target genes were differently expressed in the octopus brain masses such as in the different brain regions identified in each mass, but the most interesting results were related to the changes in the available amount of mRNA of target genes in response to fear conditioning experience.

For the sake of clarity, the results of expression level of each target gene are discussed separately in the following pages, analyzing their expression within the different regions of *O. vulgaris* brain masses and their potential involvement into the processes activated in response to fear conditioning.

ANOVA analysis has been carried out to test the effect of behavioural experience on the expression of each target gene in the brain masses and in their parts. Although the previous analyses carried out on whole brain masses showed that some target genes (*Ov-uch, Ov-TH*) were not regulated in response to behavioural experience, this ANOVA analysis showed that the target gene expression did change in some regions of brain masses in response to fear conditioning.

Table 7.6 : After MANOVA variation of target gene expression in response to the independent variables (mass, behavioural experience, regions)

Multivariate test

Independent variable	*df*	*F*	*P*
Intercept	4, 285	120.262	**< 0.0001**
Behavioural experience	4, 285	3.380	**0.0100**
Mass	8, 572	44.026	**< 0.0001**
Regions	20, 1152	3.004	**< 0.0001**
Behavioural experience * Mass	8, 572	4.791	**< 0.0001**
Behavioural experience * Regions	20, 1152	1.617	**0.0420**
Mass * Regions	28, 1152	3.167	**< 0.0001**
Behavioural experience * Mass * Regions	28, 1152	2.100	**0.0010**

7.4.1 Ubiquitin hydrolase

The expression of *Ov-uch* in each brain mass did not differ between trained and untrained octopuses. However, when its level are evaluated in different regions of each mass I recognized the lobes where *Ov-uch* was significantly up-regulated in response of learned fear.

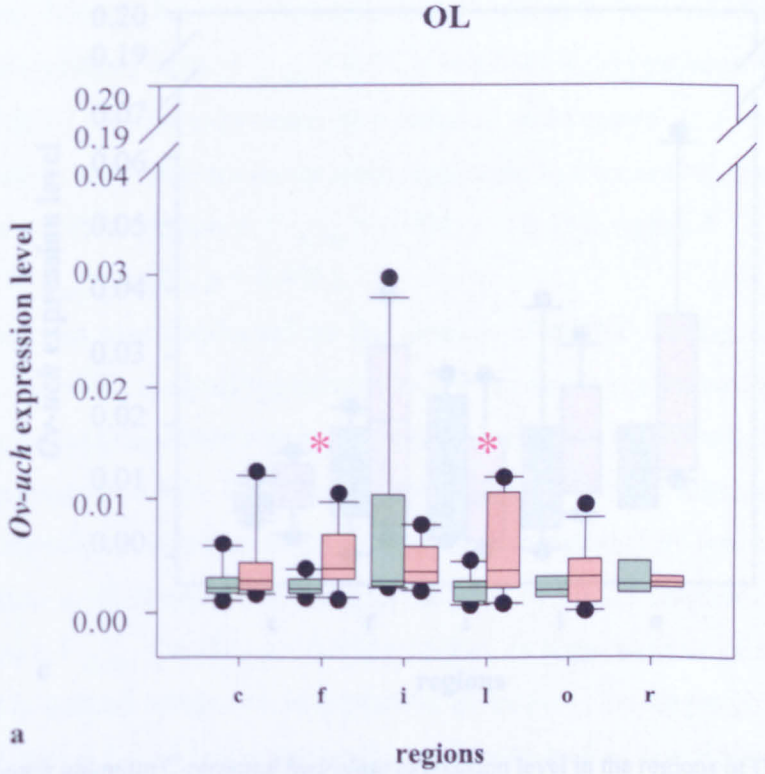
In the figure 7.9 is reported *Ov-uch* expression level in each region of OL, SEM and SUB of octopuses from both experimental groups.

In OL, a significant increase of *Ov-uch* expression has been found in regions f and l; *Ov-uch* resulted respectively 2.1 fold and 2.6 fold up-regulated in response to fear conditioning (section f: $F_{(1,\ 20)} = 7.645$, $p = 0.012$; section l: $F_{(1,\ 20)} = 5.163$, $p = 0.034$). Instead in all

other regions of OL *Ov-uch* was not differently expressed between two experimental groups (section c: $F_{(1, 20)} = 1.665$, $p = 0.212$; section i: $F_{(1, 20)} = 1.252$, $p = 0.276$; section o: $F_{(1, 18)} = 2.739$, $p = 0.115$; section r: $F_{(1, 12)} = 0.404$, $p = 0.537$).

ANOVA analysis was also conducted on the amount of mRNA coding for *Ov-uch* in SEM. Marginally significant differences have been found between the regions c of two experimental groups ($F_{(1, 20)} = 3.841$, $p = 0.064$), moreover *Ov-uch* resulted significantly up-regulated in section l of SEM in response to learned fear ($F_{(1, 20)} = 5.654$, $p = 0.027$). Thus, fear conditioning was able to induce up-regulation of *Ov-uch* allowing an increase of 4.2 times in region c and 1.8 times in region l of SEM. Instead in the regions f and i, this gene seemed expressed at the same level in the octopuses brain regardless of their behavioural experiences (region f: $F_{(1, 20)} = 1.06$, $p = 0.316$; region i: $F_{(1, 20)} = 2.820$, $p = 0.109$).

No significant differences have been found when the expression level of this gene in different regions of SUB has been compared between experimental groups (section c: $F_{(1, 20)} = 3.415$, $p = 0.079$; section f: $F_{(1, 20)} = 1.707$, $p = 0.206$; section i: $F_{(1, 20)} = 0.643$, $p = 0.432$; section l: $F_{(1, 20)} = 1.21$, $p = 0.284$; section o: $F_{(1, 18)} = 2.739$, $p = 0.115$, after ANOVA).



a

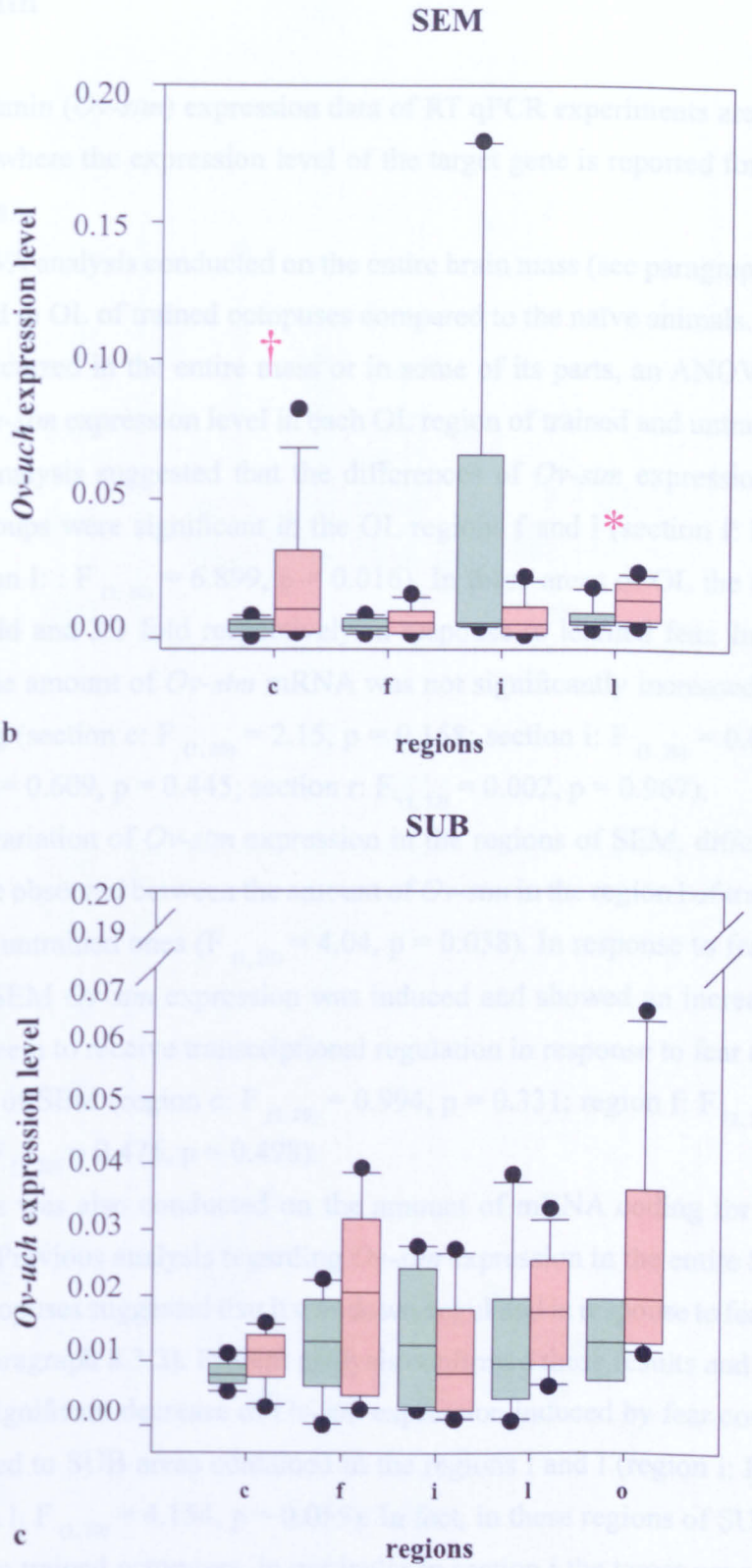


Figure 7.9 : *O. vulgaris ubiquitin C-terminal hydrolase* expression level in the regions of OL (a), SEM (b) and SUB (c). The *Ov-uch* quantity has been normalized using two reference genes: *Ov-ubi* and *Ov-tubA* as reported in materials and methods. The distribution is shown by green (naïve group) and pink (fear conditioning group) vertical box plot as median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

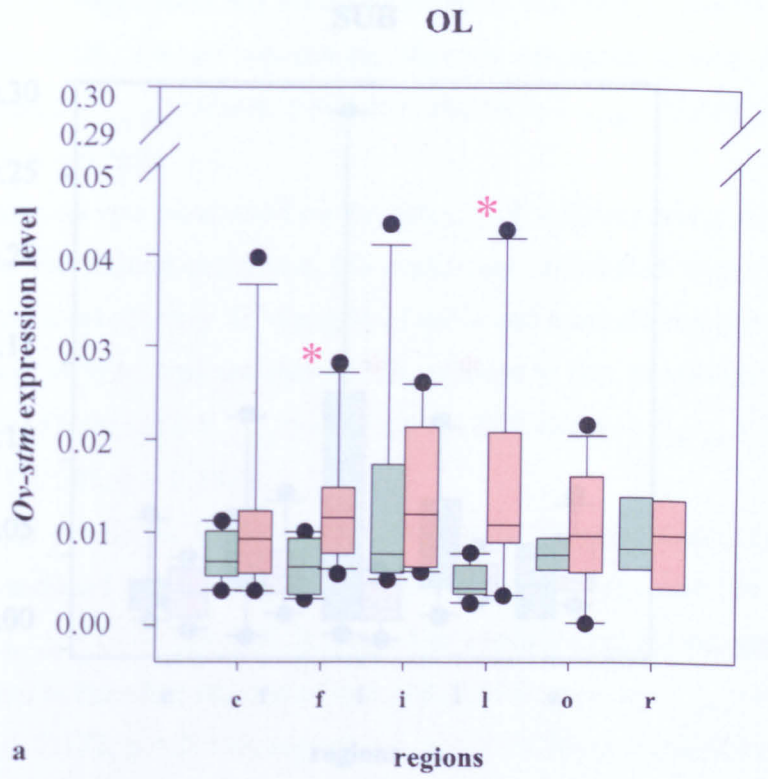
7.4.2 Stathmin

O. vulgaris stathmin (*Ov-stm*) expression data of RT qPCR experiments are summarized in the figure 7.10, where the expression level of the target gene is reported for each region of every brain mass.

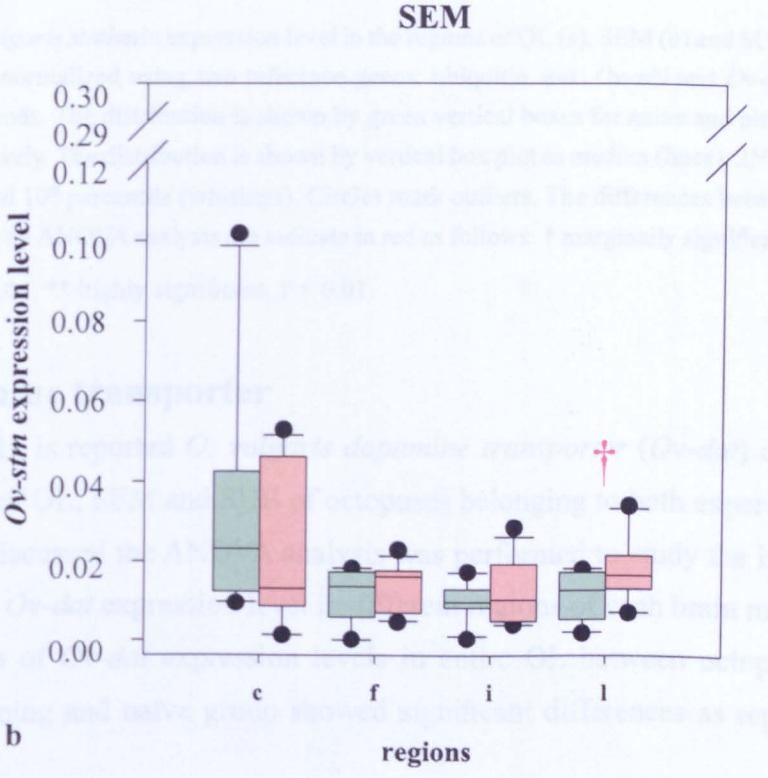
Inside the ANOVA analysis conducted on the entire brain mass (see paragraph 7.3.2) *Ov-stm* was up-regulated in OL of trained octopuses compared to the naïve animals. To study if this up-regulation occurred in the entire mass or in some of its parts, an ANOVA analysis was conducted on *Ov-stm* expression level in each OL region of trained and untrained octopuses. The statistical analysis suggested that the differences of *Ov-stm* expression between two experimental groups were significant in the OL regions f and l (section f: $F_{(1, 20)} = 7.800$, $p = 0.011$; section l: : $F_{(1, 20)} = 6.899$, $p = 0.016$). In these areas of OL the *Ov-stm* amount increased 2.1 fold and 3.3 fold respectively in response to learned fear. In the remaining regions of OL the amount of *Ov-stm* mRNA was not significantly increased in response to fear conditioning (section c: $F_{(1, 20)} = 2.15$, $p = 0.158$; section i: $F_{(1, 20)} = 0.053$, $p = 0.820$; section o: $F_{(1, 18)} = 0.609$, $p = 0.445$; section r: $F_{(1, 12)} = 0.002$, $p = 0.967$).

Looking at the variation of *Ov-stm* expression in the regions of SEM, differences close to significance were observed between the amount of *Ov-stm* in the region l of trained octopuses compared to the untrained ones ($F_{(1, 20)} = 4.04$, $p = 0.058$). In response to fear conditioning in the area l of SEM *Ov-stm* expression was induced and showed an increase of 1.6 fold. *Ov-stm* did not seem to receive transcriptional regulation in response to fear conditioning in all other regions of SEM (region c: $F_{(1, 20)} = 0.994$, $p = 0.331$; region f: $F_{(1, 20)} = 0.630$, $p = 0.436$; region i: $F_{(1, 20)} = 0.476$, $p = 0.498$).

ANOVA analysis was also conducted on the amount of mRNA coding for *Ov-stm* in the regions of SUB. Previous analysis regarding *Ov-stm* expression in the entire SUB of trained and untrained octopuses suggested that it was down-regulated in response to fear conditioning (as reported in paragraph 8.3.2). Present analysis confirmed these results and suggested that this marginally significant decrease of *Ov-stm* expression induced by fear conditioning was particularly related to SUB areas contained in the regions i and l (region i: $F_{(1, 20)} = 4.232$, $p = 0.053$; region l: $F_{(1, 20)} = 4.154$, $p = 0.055$). In fact, in these regions of SUB *Ov-stm* was down-regulated in trained octopuses, in particular in section i the target gene was 3.3 times and in regions l was 1.5 fold less expressed in response to fear conditioning. In the other regions of SUB *Ov-stm* did not appear significantly neither down-regulated or up-regulated in response of learned fear (section c: $F_{(1, 20)} = 0.028$, $p = 0.870$; section f: $F_{(1, 20)} = 0.952$, $p = 0.341$; section o: $F_{(1, 18)} = 0.609$, $p = 0.445$).


OL
0.30
0.29
0.05
0.04
0.03
0.02
0.01
0.00
Ov-stm expression level
*
*
c
f
i
l
o
r
regions

a


SEM
0.30
0.29
0.12
0.10
0.08
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0.04
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0.00
Ov-stm expression level
†
c
f
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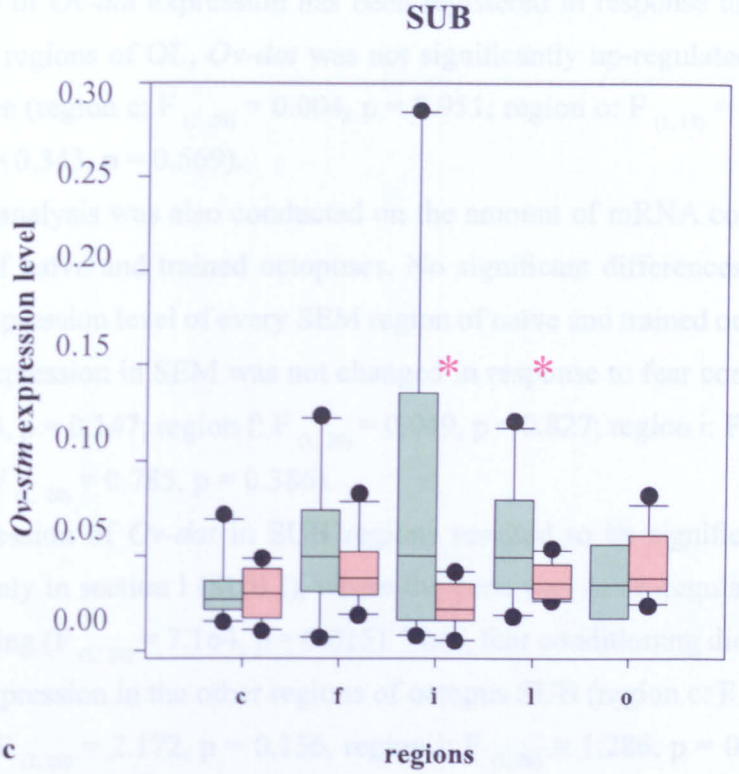
regions

b



c

Figure 7.10 : *O. vulgaris stathmin* expression level in the regions of OL (a), SEM (b) and SUB (c). The *stathmin* quantity has been normalized using two reference genes: ubiquitin and *Ov-ubi* and *Ov-tubA* as reported in materials and methods. The distribution is shown by green vertical boxes for naive and pink boxes for trained octopuses, respectively. The distribution is shown by vertical box plot as median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

7.4.3 Dopamine transporter

In the figure 7.11 is reported *O. vulgaris dopamine transporter* (*Ov-dat*) expression level in every region of OL, SEM and SUB of octopuses belonging to both experimental groups. As previously discussed the ANOVA analysis was performed to study the influence of fear conditioning on *Ov-dat* expression level in different regions of each brain mass.

The comparison of *Ov-dat* expression levels in entire OL between octopuses belonging to fear conditioning and naïve group showed significant differences as reported above in paragraph 7.3.3.

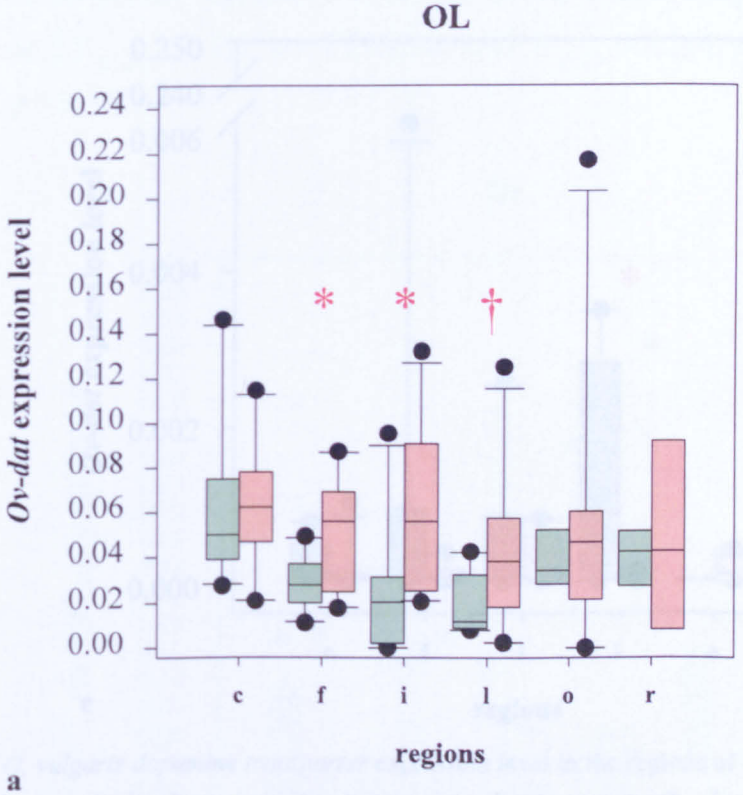
To study if these differences are conserved in all OL regions, an ANOVA analysis has been conducted on *Ov-dat* expression level in each OL region of trained and untrained octopuses. The statistical analysis suggested that the differences of *Ov-dat* expression between two experimental groups were significant or marginally significant in the OL regions f, i and l (region f: $F_{(1, 20)} = 7.437$, $p = 0.013$; region i: $F_{(1, 20)} = 6.697$, $p = 0.018$; region l: $F_{(1, 20)} = 4.350$, $p = 0.05$).

In these areas of OL an increase of 1.8 times (region f) or 2.6 times (region i) or 2.4 times

(region l) of *Ov-dat* expression has been registered in response to learned fear. Instead in the other regions of OL, *Ov-dat* was not significantly up-regulated after fear conditioning experience (region c: $F_{(1, 20)} = 0.004$, $p = 0.951$; region o: $F_{(1, 18)} = 0.229$, $p = 0.638$; region r: $F_{(1, 12)} = 0.343$, $p = 0.569$).

ANOVA analysis was also conducted on the amount of mRNA coding for *Ov-dat* in SEM regions of naïve and trained octopuses. No significant differences were found comparing *Ov-dat* expression level of every SEM region of naïve and trained octopuses, confirming that *Ov-dat* expression in SEM was not changed in response to fear conditioning (region c: $F_{(1, 20)} = 2.273$, $p = 0.147$; region f: $F_{(1, 20)} = 0.049$, $p = 0.827$; region i: $F_{(1, 20)} = 0.687$, $p = 0.417$; region l: $F_{(1, 20)} = 0.785$, $p = 0.386$).

The expression of *Ov-dat* in SUB regions resulted to be significantly different between groups only in section l (SUB l), where the gene was down-regulated (30 times) after fear conditioning ($F_{(1, 20)} = 7.164$, $p = 0.015$). Thus, fear conditioning did not appear to affect the *Ov-dat* expression in the other regions of octopus SUB (region c: $F_{(1, 20)} = 0.504$, $p = 0.486$; region f: $F_{(1, 20)} = 2.172$, $p = 0.156$, region i: $F_{(1, 20)} = 1.286$, $p = 0.270$; region o: $F_{(1, 20)} = 0.229$, $p = 0.638$).



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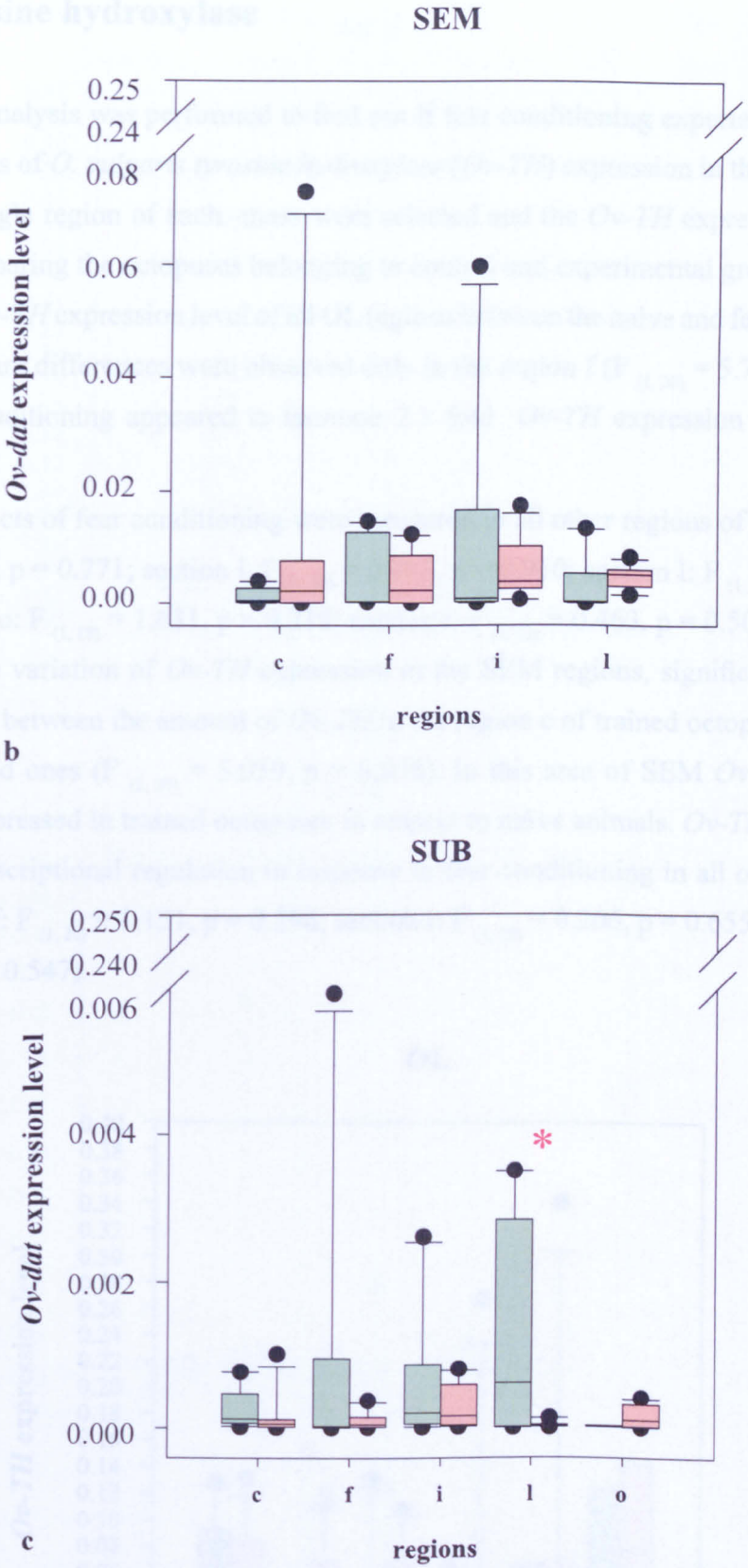


Figure 7.11: *O. vulgaris dopamine transporter* expression level in the regions of OL (a), SEM (b) and SUB (c). The *Ov-dat* quantity has been normalized using two reference genes: *Ov-ubi* and *Ov-tubA* as reported in materials and methods. The distribution is shown by green vertical boxes for naive and pink boxes for trained octopuses, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

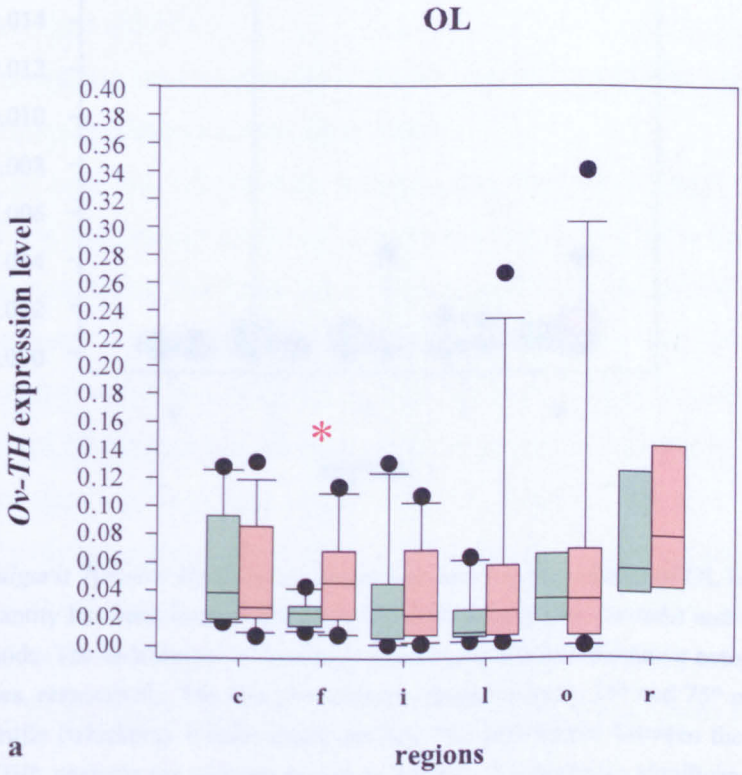
7.4.4 Tyrosine hydroxylase

An ANOVA analysis was performed to find out if fear conditioning experience was able to induce changes of *O. vulgaris tyrosine hydroxylase* (*Ov-TH*) expression in the octopus brain lobes. The single region of each mass were selected and the *Ov-TH* expression level was analyzed comparing the octopuses belonging to control and experimental group.

Comparing *Ov-TH* expression level of all OL regions between the naive and fear conditioning group significant differences were observed only in the region f ($F_{(1, 20)} = 5.745$, $p = 0.026$). Thus, fear conditioning appeared to increase 2.1 fold *Ov-TH* expression in this area of octopus OL.

Instead no effects of fear conditioning were measured in all other regions of OL (section c: $F_{(1, 20)} = 0.087$, $p = 0.771$; section i: $F_{(1, 20)} = 0.013$, $p = 0.910$; section l: $F_{(1, 20)} = 2.243$, $p = 0.150$; section o: $F_{(1, 18)} = 1.631$, $p = 0.218$; section r: $F_{(1, 12)} = 0.463$, $p = 0.509$).

Looking at the variation of *Ov-TH* expression in the SEM regions, significant differences were observed between the amount of *Ov-TH* in the region c of trained octopuses in respect to the untrained ones ($F_{(1, 20)} = 5.039$, $p = 0.036$). In this area of SEM *Ov-TH* resulted 3 times more expressed in trained octopuses in respect to naïve animals. *Ov-TH* did not seem to receive transcriptional regulation in response to fear conditioning in all other regions of SEM (section f: $F_{(1, 20)} = 1.151$, $p = 0.296$; section i: $F_{(1, 20)} = 0.206$, $p = 0.655$; section l: $F_{(1, 20)} = 0.376$, $p = 0.547$).



a

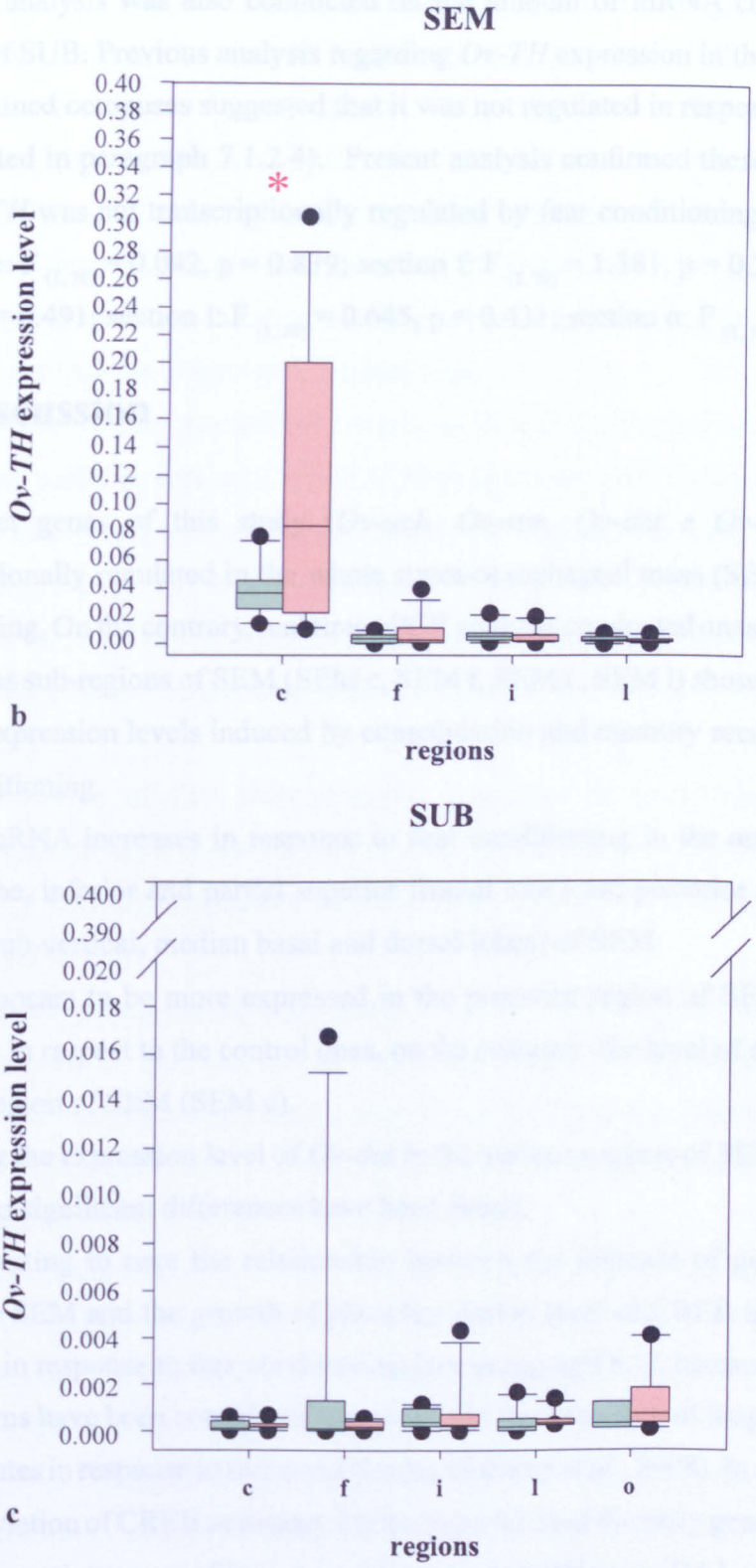


Figure 7.12 : *O. vulgaris Tyrosine Hydroxylase* expression level in the regions of OL (a), SEM (b) and SUB (c). The *Ov-TH* quantity has been normalized using two reference genes: *Ov-tubA* and *Ov-ubi* as reported in materials and methods. The distribution is shown by green vertical boxes for naive octopuses and pink boxes for trained octopuses, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

ANOVA analysis was also conducted on the amount of mRNA coding for *Ov-TH* in all regions of SUB. Previous analysis regarding *Ov-TH* expression in the entire SUB of trained and untrained octopuses suggested that it was not regulated in response to fear conditioning (as reported in paragraph 7.1.2.4). Present analysis confirmed these results and suggested that *Ov-TH* was not transcriptionally regulated by fear conditioning in any region of SUB (section c: $F_{(1, 20)} = 0.042$, $p = 0.839$; section f: $F_{(1, 20)} = 1.381$, $p = 0.254$; section i: $F_{(1, 20)} = 0.493$, $p = 0.491$; section l: $F_{(1, 20)} = 0.645$, $p = 0.431$; section o: $F_{(1, 18)} = 1.631$, $p = 0.218$).

7.5 Discussion

The target genes of this study (*Ov-uch, Ov-stm, Ov-dat e Ov-TH*) seem not to be transcriptionally regulated in the whole supra-oesophageal mass (SEM) in response to fear conditioning. On the contrary, real time qPCR analysis conducted on target gene expression in the various sub-regions of SEM (SEM c, SEM f, SEM i , SEM l) show significant differences of gene expression levels induced by consolidation and memory recall processes following fear conditioning.

Ov-uch mRNA increases in response to fear conditioning in the anterior (region SEM c: buccal lobe, inferior and partial superior frontal lobe) and posterior region (region SEM l: vertical, sub-vertical, median basal and dorsal lobes) of SEM.

Ov-stm appears to be more expressed in the posterior region of SEM (SEM l) of trained octopuses in respect to the control ones, on the contrary the level of *Ov-TH* increases in the anterior region of SEM (SEM c).

Analyzing the expression level of *Ov-dat* in the various regions of SEM of trained and naive animals no significant differences have been found.

It is interesting to note the relationship between the increase of gene expression of *Ov-uch* in the SEM and the growth of phosphorylation level of CREB in the same brain mass measured in response to fear conditioning (see paragraph 6.1), because these two molecular mechanisms have been considered necessary for the formation of long term memory (LMT) in vertebrates in response to fear conditioning (Sakurai *et al.*, 2008). In addition, the persistent phosphorylation of CREB necessary for the expression of the early genes (i.e. *uch*) is required also for the maintenance of long term potentation (LTP) in the CA1 area of hippocampus of vertebrates after avoidance test (Impey *et al.*, 1998). A form of LTP is induced in response to fear conditioning in the vertical lobe of octopus (Shomrat *et al.*, 2008). Thus, it seems to be possible to establish an analogy between vertebrates and invertebrates, because either induce LTP formation, CREB phosphorylation and early gene expression (*uch*) in response to fear conditioning.

Also the increase of *Ov-stm* expression in the posterior region of SEM in response to learned

fear suggests a similarity between vertebrate and invertebrate organisms.

In fact it is known the key role of stathmin in the induction of LTP in the amygdala and in the regulation of learned fear in vertebrates. The increase of stathmin expression is measured in the amygdala of animals subjected to fear conditioning, on the other hand the disruption of stathmin expression in the amygdala cause impairment in the formation of LTP and of fear-related memory. (Shumyatsky *et al.*, 2005). It is possible to suppose that stathmin such as in the vertebrates also in octopus is able to play a regulatory role in the formation of LTP and LTM activated in response to learned fear.

In these processes, the vertical lobe seems to have a leading role together with other lobes lying in the posterior region of the SEM, they appear to play a role similar to that played by amygdala and limbic system of vertebrates in the formation of LTM fear-related.

Finally, the increase of *Ov-TH* expression in the anterior region of SEM and the current absence of variation of *dat* expression level let to suppose the involvement of noradrenaline (NA) in the process of reconsolidation and recall of LTM activated in response to fear conditioning.

The involvement of this catecholamine is known in vertebrates where genetic and pharmacological approaches reveal the role played by NA system in the regulation of processes of learning and memorization induced by conditioned learning tasks (Kobayashi and Kobayashi, 2001).

Analyzing the expression of target genes in the sub-oesophageal mass (SUB) of naive and trained animals, *Ov-stm* and *Ov-dat* result the genes that are subjected to transcriptional regulation in response to fear conditioning. *Ov-stm* is down-regulated in the median-posterior regions of SUB (region SUB i : pedal lobe; SUB l: partial palliovisceral lobe, vasomotor and posterior cromatophore lobes) of trained octopuses.

The decreased expression of stathmin, that is negative regulator of microtubule formation, should have to assure the increase of microtubule quantity and half life, thus regulating the synaptic plasticity and activity of cells lying in the median-posterior lobes of SUB of trained octopuses (Shumyatsky *et al.*, 2005).

Ov-dat is down-regulated in the most posterior lobes of the SUB, in particular in the posterior pedal, magnocellur and palliovisceral lobes. Considering that in the SUB is not registered a decrease of *TH* expression, it could imagine that a decrease of *dat* expression is indicative of an involvement of noradrenergic neurons instead of dopaminergic ones in the processes activated in response to fear conditioning.

Ov-stm and *Ov-dat* are up-regulated in the optic lobes (OL) of trained octopuses compared to the control ones. Also the other target genes seem to be up-regulated in some sub-regions of OL (OL c, f, i, l, o, r) in response to fear conditioning.

In fact, *Ov-uch* and *Ov-stm* expression levels increase in the regions f (composed by: medulla,

inner and outer layer), and l (composed by: medulla, inner and outer layer, peduncle lobe and optic gland) of trained octopuses. *Ov-dat* is up-regulated besides in the regions f and l also in the region i (composed by: medulla, inner and outer layer, olfactory and peduncle lobes). On the contrary *Ov-TH* mRNA is increased only in the region f of trained octopuses' OL.

It is interesting to note that all these genes, known to be involved in the processes of memory activated in response to fear conditioning in vertebrates, are subject to transcriptional regulation in response to learned fear in the optic lobes of octopus. These octopus brain masses are considered the site of memory storage (Young, 1962; Young, 1971), for this reason the change of target genes' expression in this district of CNS suggests that these genes could be involved in the formation of LTM also in octopus brain.

CHAPTER 8

INNATE FEAR IN *OCTOPUS VULGARIS*: MOLECULAR ANALYSIS OF THE CIRCUITRY

8.1 Analysis of gene expression in response to innate fear

To study the relationship between social interaction and gene expression in octopus, a solitary animal, I applied real-time quantitative PCR experiments on samples taken from acclimated animals and octopuses exposed to interaction with a conspecific. As reported in the previous sections of this thesis I used a biased approach to select genes used as target and, together with the genes previously reported (Ubiquitin hydrolase, stathmin, tyrosine hydroxylase and dopamine transporter), I studied also the expression of other two genes octopressin and cephalotocin. These have been selected on the basis of the available information on their involvement in processes activated in response to social learning and interaction. Octopressin and cephalotocin are homologs of vasopressin (AVP) and oxytocin (OT), members of a large group of ancient neuropeptides that have profound effects on a variety of mnemonic and social processes in vertebrates. Social memory is a unique form of memory, critical for reproduction, territorial defense, and the establishment of dominance hierarchies in nature. AVP and OT have been shown to influence a number of forms of social behavior, including affiliation, aggression, and reproduction (for review see Gulpinar and Yegen, 2004). I studied the expression of octopressin and cephalotocin in response to social interaction to understand if these two genes could play in octopus the same role that AVP and OT have in vertebrates.

8.2 Materials and methods

8.2.1 Subjects

A total of 11 *Octopus vulgaris* of both sexes weighting from 200 to 400 g were caught in the Bay of Napoli (Italy) during the summer 2007. The octopuses were randomly assigned to control (sacrificed after 4 days of acclimatization to laboratory conditions; Acclimatized, N = 6) and experimental group (sacrificed after 4 days of social interaction with a conspecific; Social, N = 5).

8.2.2 Samples-acclimatization and social interaction

After dissection, the different part of the brain (OL, SEM, SUB) were placed in plastic moulds (Peel – A – Way Disposable Embedding Molds 22 x 22 mm Polyscience Inc.-Warrington PA USA), immediately frozen in liquid nitrogen and stored at -80°C until processed.

8.2.3 RNA isolation and quantification

The brain masses were supplemented with 1 ml of buffer Eurozol (EuroClone, Pavia, Italy) and homogenized with homogenizer (Ultra-turrax T25: J&K-IKA Labortechnik, Staufen, Germany). The procedures used to isolate, purify and quantify the RNA were above described (see paragraph 7.2.3).

8.2.4 cDNA synthesis

cDNA was synthesized using 0.5 μg of total RNA per sample. The procedure employed to synthesized cDNA is above described (see paragraph 7.2.4).

8.2.5 Real-time qPCR

Two μl of diluted cDNA were used in a SYBR Green PCR for each reaction. Polymerase chain reactions were carried out in an optical 96-well plate with a Chromo4™ Real-Time Detector (BioRad, Hercules, CA) thermal cycler using FastStart SYBR Green Master mix (Roche, Indianapolis, IN) to monitor dsDNA synthesis. Reactions (total volume: 10 μl) contained: 2 μl cDNA, 2.5 μl SYBR Green Master mix reagent, 0.3 μM (each) of forward and reverse primers. The thermal profile used is above reported (see paragraph 7.2.5). Fluorescence was measured using Opticon Monitor 3.1 (BioRad, Hercules, CA). Each assay included a no-

template control for every primer pair and a standard curve with 1:10, 1:25, 1:50, 1:100, 1:200 dilutions of the standard cDNA. The standard sample was a pool of equal amount of 6 randomly chosen samples belonging to the three octopus brain masses. To capture inter-assay variability all RT qPCR plates contained inter-run calibrators.

8.2.6 Primer design: efficiency and specificity

Primers were designed by Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) using sequences for specific octopus mRNA (table 8.1). Primer parameter setting is above described (see paragraph 7.2.6), in the following table is reported only the sequences, the amplicon size and the efficiency of primers for cephalotocin and octopressin, not listed above.

Table 8.1: Primers sequences, amplicons size and amplification efficiency of target genes.

Gene	GenBank accession number	Gene Ontology*	Primer	Primer sequence 5' - 3'	Amplicon size (bp)	Efficiency
Ov - CT	AB162925	Hormone activity (Fu) GO:0005179	F	TCGTCCAACTTTTGTTCGTG	119	1.7
			R	CCTGCTCTGACATGGGTGT		
Ov OP	AB162924	Hormone activity (Fu) GO:0005179	F	TCCCAAAAAGTTCACAAATCAA	123	1.8
			R	ATAGGGCAGCTTGTCCAGAA		

* The biological function (Fu), biological process (P), cellular components (C) accompanied by a GO number has been listed for each gene according to http://www.geneontology.org/

The efficiency for each pair of primers has been calculated as reported above, such as for melting curve and PCR product analysis (see paragraph 7.2.6).

8.2.7 Reference genes and normalization

As internal control genes have been used the reference genes positively selected previously in Sirakov *et al.* (2009): *Ov-tubA* and *Ov-ubi/S27A*).

The gene stability analysis was conducted using the geNorm software as above reported (see paragraph 7.2.7; for details on study of stability of candidate reference genes see Appendix 4).

8.3 Results: target genes expression in the masses of *O. vulgaris* CNS

Quantitative real-time PCR has been used to study the expression of six genes of interest in the octopus brain masses (SEM, SUB, OL) in response to social interaction.
In order to identify the transcriptional regulation of target genes induced by behavioural experiences, the expression level of each gene has been studied in the brain of six octopuses of control group and five octopuses of social interaction group. The brains have been dissected and the RNA of each brain mass was analyzed as described in materials and methods.
A multivariate test was conducted to consider the relationship between variation of gene expression (dependent variables) and other factors (independent variables) such as brain mass and behavioural experience. The target genes were differentially expressed in the octopus brain masses and they are regulated in different way in response to the behavioural experiences as reported in the table reported below (table 8.2).

Table 8.2 After MANOVA variation of target gene expression in response to the independent variables (behavioural experience and mass).

Multivariate test

Indipendent variable	*df*	*F*	*P*
Intercept	6, 55	228.825	**< 0.0001**
Behavioural experience	6, 55	4.446	**< 0.0001**
Mass	12, 112	34.453	**0.001**
Behavioural experience x Mass	12, 112	2.285	**0.012**

A MANOVA analysis has been conducted to test if the target gene expression changed in each mass in response to the behavioural experience. The results of this analysis are reported in the table below (table 8.3) and suggest that in the OL the amount of mRNA of the target genes changed in response to social interaction, whereas no significant differences were detected in the SEM and SUB between octopuses subjected to social interaction and control group.

Table 8.3 After MANOVA variation of target gene expression in each mass in response to behavioural experience.

Multivariate test

	OL			*SEM*			*SUB*		
Indipendent variable	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Intercept	6, 15	385.432	**<0.0001**	6, 15	55.979	**<0.0001**	6, 15	75.212	**<0.0001**
Behavioural experinece	6, 15	3.385	**0.026**	6, 15	2.236	0.097	6, 15	1.907	0.146

In the following pages, I have analyzed the expression level of six target genes (ubiquitin hydrolase, stathmin, dopamine transporter, Tyrosine Hydroxylase, octopressin and

cephalotocin) within the different masses of the *O. vulgaris* central nervous system and their putative involvement in the processes activated in response to social interacion. For sake of clarity, expression data of each target gene are discussed separately.

8.3.1 Ubiquitin C-terminal hydrolase

O. vulgaris ubiquitin C-terminal hydrolase (*Ov-uch*) expression data are summarized in figure 8.1. Analyzing the *Ov-uch* expression in OL, in SEM and in SUB suggested difference between the masses in each experimental group and this observation was confirmed by ANOVA analysis and post hoc Bonferroni test ($F(2, 35) = 31.028$, $p < 0.0001$ for acclimatization group; $F(2, 29) = 15.915$, $p < 0.0001$ for social interaction group). In particular, in both experimental groups *Ov-uch* was more expressed in SUB compared to OL and SEM, even if the significant or marginally significant differences were present between each mass (OL vs SEM $p = 0.067$, OL vs SUB $p < 0.0001$, SEM vs SUB $p < 0.0001$ for acclimatization group; OL vs SEM $p = 0.031$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 0.023$, for social interaction group).

In order to analyze the effect of social interaction on *Ov-uch* expression, ANOVA analysis was conducted. The target gene expression in OL was significantly changed by behavioural experience ($F_{(1, 21)} = 7.336$, $p = 0.014$). In contrast, no significant differences were found in the *Ov-uch* expression in SEM ($F_{(1, 21)} = 2.410$, $p = 0.136$) and SUB ($F_{(1, 21)} = 0.529$, $p = 0.475$). These results suggested that *Ov-uch* is transcriptionally regulated in response to social interaction only in the OL, where it could be involved in the molecular mechanisms regulating innate fear.

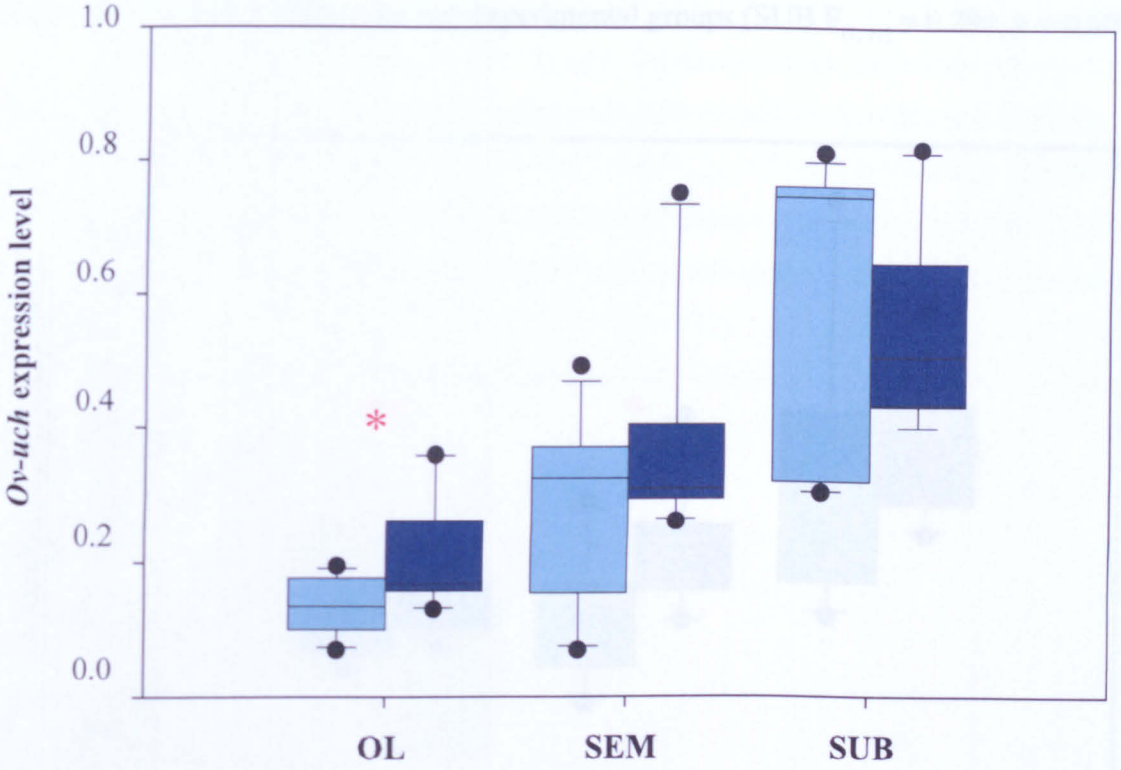


Figure 8.1: *ubiquitin C-terminal hydrolase* expression level in OL, SEM and SUB. The *Ov-uch* quantity has been normalized using two reference genes: *Ov-ubi* and *Ov-tubA*. The distribution is shown by cyan vertical boxes for acclimatization group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

8.3.2 Stathmin

O. vulgaris stathmin (*Ov-stm*) expression data are summarized in the figure 8.2. *Ov-stm* was differently expressed in the brain masses of each experimental group using ANOVA analysis and Bonferroni post hoc test ($F(2, 35) = 14.863$, $p < 0.0001$ for acclimatization group; $F(2, 29) = 19.891$, $p < 0.0001$ for social interaction group). In particular, in both experimental groups significant differences were found between *Ov-stm* expression in SUB compared to all other masses (OL vs SEM p = 1.000, OL vs SUB p < 0.0001, SEM vs SUB p < 0.0001 for acclimatization group; OL vs SEM p = 0.138, OL vs SUB p < 0.0001, SEM vs SUB p = 0.001 for social interaction group).

Social interaction induced changes in OL *Ov-stm* expression, because the target gene was significantly more expressed in the OL of octopuses subjected to social interaction compared to acclimated ones ($F_{(1, 21)} = 13.189$, $p = 0.002$).

Also in SEM a significant increase of *Ov-stm* expression level was revealed in response to social interaction, confirming that the behavioural experience seemed to induce changes in the *Ov-stm* mRNA availability ($F_{(1, 21)} = 5.934$, $p = 0.024$).

In contrast, no significant differences emerged when the *Ov-stm* expression level was

compared between SUBs of the two experimental groups (SUB $F_{(1, 21)} = 0.284$, $p = 0.600$).

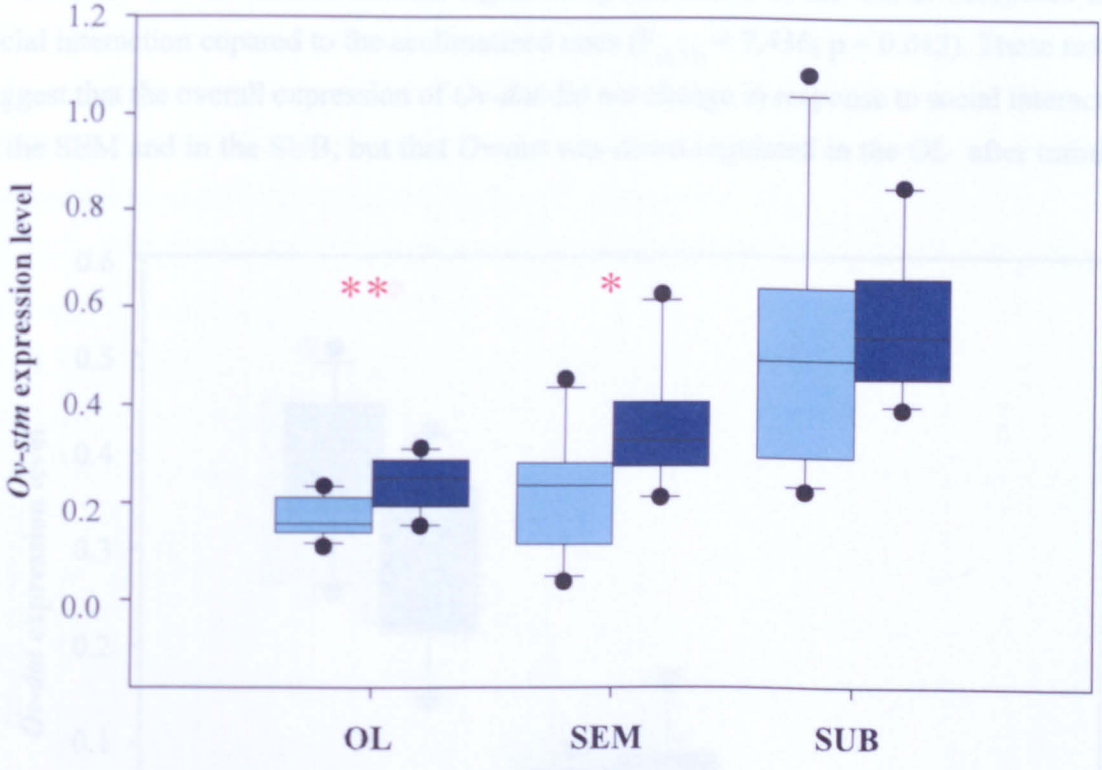


Figure 8.2 : *stathmin* expression level in SEM, SUB and OL. *Ov-stm* quantity are normalized using normalization factor generated by GeNorm with two reference genes *Ov-tubA* and *Ov-ubi*. The distribution is shown by cyan vertical boxes for acclimatization group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

8.3.3 Dopamine transporter

O. vulgaris dopamine transporter (*Ov-dat*) expression data were summarized in figure 8.3. *Ov-dat* was not equally expressed in the octopus CNS, but its expression level showed significant differences between the brain masses of each experimental group as suggested by ANOVA analysis and Bonferroni post hoc test ($F_{(2, 35)} = 249.972$, $p < 0.0001$ for acclimatization group; $F_{(2, 29)} = 66.722$, $p < 0.0001$ for social interaction group). In particular *Ov-dat* expression in the OL was significantly higher compared to the other masses in both acclimated animals (OL vs SEM $p < 0.0001$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 0.050$) and octopuses subjected to social interaction (OL vs SEM $p < 0.0001$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 0.120$). In order to analyze if the behavioural experience was the factor that influenced *Ov-dat* expression in each brain mass an ANOVA analysis was conducted. The expression level of *Ov-dat* was considered separately for each brain mass. No significant differences of *Ov-*

dat mRNA amount were found in SEM ($F_{(1, 21)} = 0.188$, $p = 0.669$) and SUB ($F_{(1, 21)} = 2.717$, $p = 0.115$) comparing the experimental groups (acclimatization and social interaction).In contrast, the *Ov-dat* mRNA amount significantly decreased in the OL of octopuses after social interaction copared to the acclimatized ones ($F_{(1, 21)} = 7.436$, $p = 0.013$). These results suggest that the overall expression of *Ov-dat* did not change in response to social interaction in the SEM and in the SUB, but that *Ov-dat* was down-regulated in the OL after training.

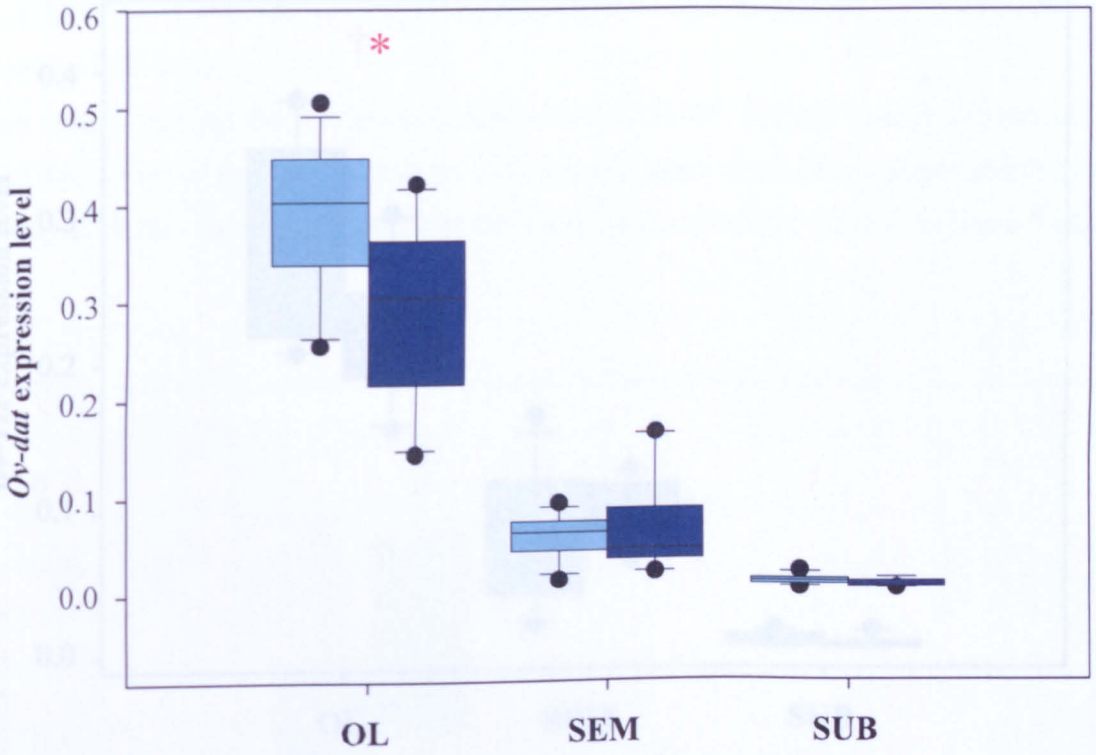


Figure 8.3: *dopamine transporter* expression level in SEM, SUB and OL. *Ov-dat* quantity are normalized using normalization factor generated by GeNorm with two reference genes *Ov-tubA* and *Ov-ubi*. The distribution is shown by cyan vertical boxes for acclimatization group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01

8.3.4 Tyrosine hydroxylase

The figure 8.4 summarized the *O. vulgaris* tyrosine hydroxylase (*Ov-TH*) expression data. *Ov-TH* was differentially expressed in the brain masses of acclimated octopuses ($F_{(2, 35)} = 95.933$, $p < 0.0001$) and animals subjected to social interaction ($F_{(2,29)} = 154.054$, $p < 0.0001$). The expression levels of *Ov-TH* were significantly higher in OL in comparison with other brain masses in both control (OL vs SEM $p < 0.0001$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 0.001$) and experimental group (OL vs SEM $p < 0.0001$, OL vs SUB $p < 0.0001$, SEM vs

SUB $p < 0.0001$). In order to analyze the effect of social interaction on *Ov-TH* expression an ANOVA analysis was conducted comparing *Ov-TH* expression level of each mass between the two experimental groups. Social interaction induced only marginally significant changes of the *Ov-TH* expression in the OL ($F_{(1, 21)} = 3.831$, $p = 0.064$), while this experience did not induce significant changes neither in SEM ($F_{(1, 21)} = 0.801$, $p = 0.381$) or in SUB ($F_{(1, 21)} = 2.989$, $p = 0.099$). Thus, these results suggest that overall *Ov-TH* expression was decreased in response to social interaction only in the OL of octopus CNS.

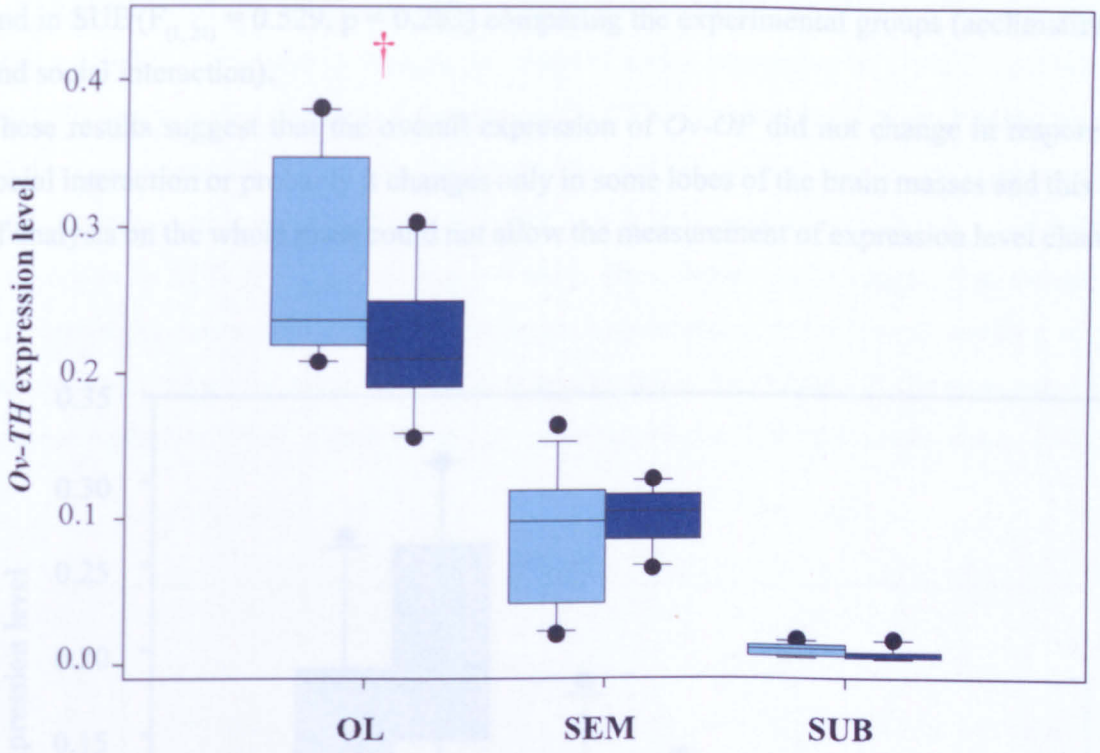


Figure 8.4 : *Tyroine Hydroxylase* expression level in SEM, SUB, OL. Quantity data are normalized using normalization factor generated by GeNorm using two reference genes *Ov-tubA* and *Ov-ubi*. The distribution is shown by cyan vertical boxes for acclimatization group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups that resulted significant by ANOVA analysis are indicate in red as follows: † marginally significant, P = 0.05 – 0.07; * significant, P < 0.05; ** highly significant, P < 0.01.

8.3.5 Octopressin

O. vulgaris octopressin (*Ov-OP*) expression data are summarized in figure 8.5. *Ov-OP* was not equally expressed in the octopus CNS, but its expression level showed significant differences between the brain masses of each experimental group as suggested by ANOVA analysis and Bonferroni post hoc test ($F_{(2, 35)} = 31.028$, $p < 0.0001$ for acclimatization group; $F_{(2, 29)} = 36.129$, $p < 0.0001$ for social interaction group). In particular *Ov-OP* expression in the OL was significantly higher compared to the other masses in both acclimated animals (OL vs SEM $p = 0.003$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 0.004$) and octopuses

subjected to social interaction (OL vs SEM $p < 0.0001$, OL vs SUB $p < 0.0001$, SEM vs SUB $p = 0.038$). Amounts of OP mRNA were high in the supraesophageal brain and low in the subesophageal brain confirming the *in situ* results of Takura-Kuroda and coworkers (2003). In order to analyze if the behavioural experience was the factor that influenced *Ov-OP* expression in each brain mass an ANOVA analysis was conducted. The expression level of *Ov-OP* was considered separately for each brain mass. No significant differences of *Ov-OP* mRNA amount were found in OL ($F_{(1,21)} = 7.336$, $p = 0.102$) in SEM ($F_{(1,21)} = 2.410$, $p = 0.538$) and in SUB ($F_{(1,21)} = 0.529$, $p = 0.283$) comparing the experimental groups (acclimatization and social interaction).

These results suggest that the overall expression of *Ov-OP* did not change in response to social interaction or probably it changes only in some lobes of the brain masses and this type of analysis on the whole mass could not allow the measurement of expression level changes.

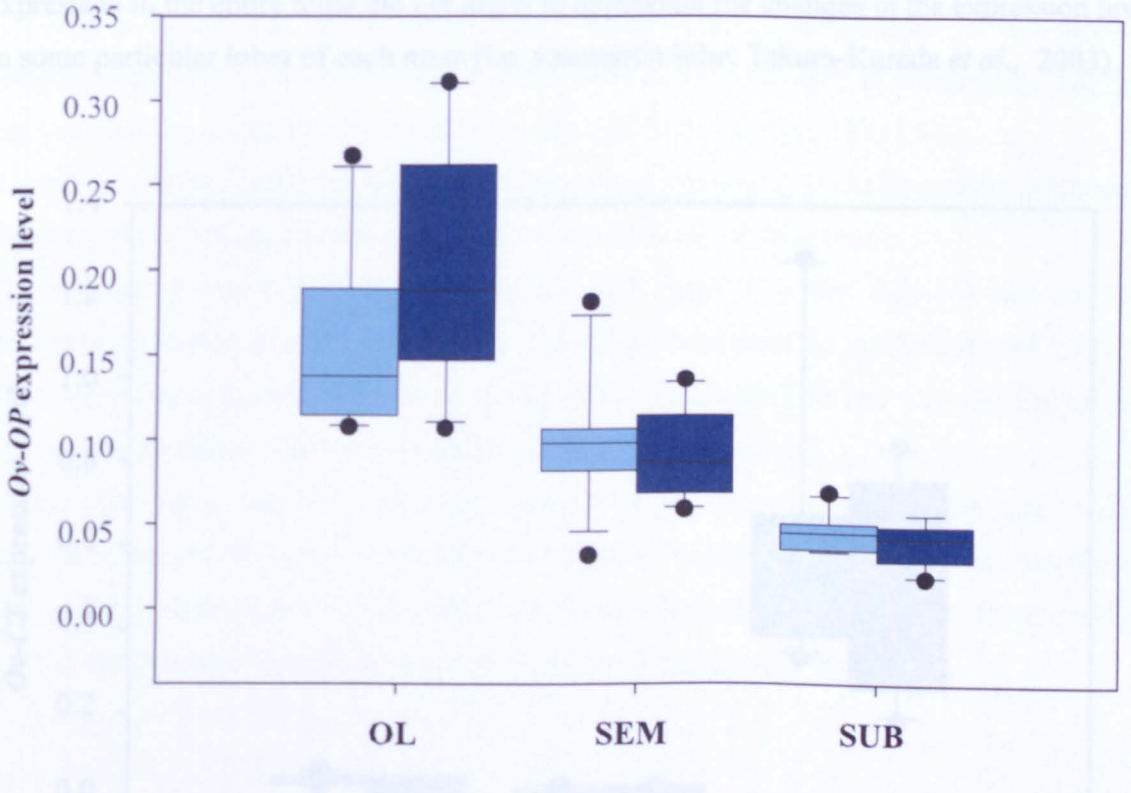


Figure 8.5: *Octopressin* expression level in SEM, SUB, OL. Quantity data are normalized using normalization factor generated by GeNorm using two reference genes *Ov-tubA* and *Ov-ubi*. The distribution is shown by cyan vertical boxes for acclimatization group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups resulted no significant by ANOVA analysis.

8.3.6 Cephalotocin

The figure 8.6 summarized the *O. vulgaris* cephalotocin (*Ov-CT*) expression data. *Ov-CT* was differentially expressed in the brain masses of acclimated octopuses ($F_{(2, 35)} = 35.230$, $p < 0.0001$) and animals subjected to social interaction ($F_{(2, 29)} = 39.865$, $p < 0.0001$). The expression levels of *Ov-CT* were significantly higher in SUB in comparison with other brain masses in both control (OL vs SEM $p = 1.000$, OL vs SUB $p < 0.0001$, SEM vs SUB $p < 0.0001$) and experimental groups (OL vs SEM $p = 1.000$, OL vs SUB $p < 0.0001$, SEM vs SUB $p < 0.0001$). In order to analyze the effect of social interaction on *Ov-CT* expression an ANOVA analysis was conducted comparing *Ov-CT* expression level of each mass between the two experimental groups. Social interaction did not induce significant changes of the *Ov-CT* expression in the OL ($F_{(1, 21)} = 1.399$, $p = 0.251$), neither in SEM ($F_{(1, 21)} = 0.030$, $p = 0.864$) or in SUB ($F_{(1, 21)} = 0.519$, $p = 0.480$). Thus, these results suggest that overall *Ov-CT* expression has no effect in response to social interaction or the overall analysis of gene expression in the entire mass did not allow to appreciate the changes in the expression level in some particular lobes of each mass (i.e. vasomotor lobe; Takura-Kurada *et al.*, 2003).

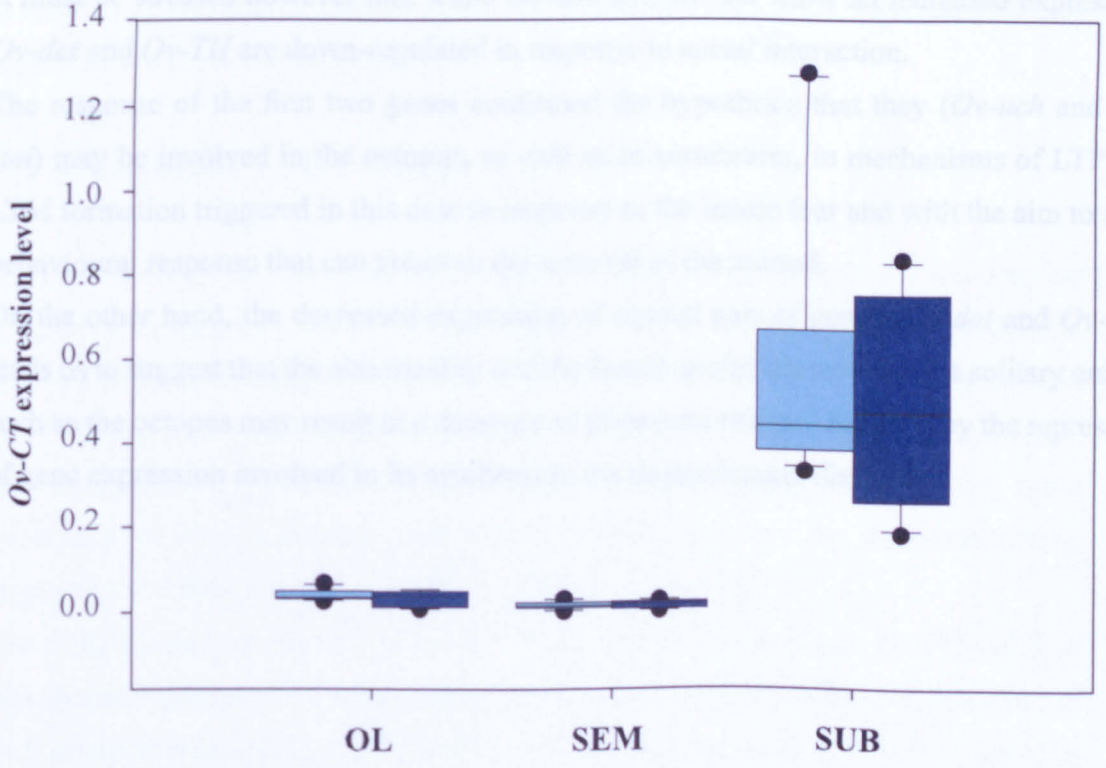


Figure 8.6 : *Cephalotocin* expression level in SEM, SUB, OL. Quantity data are normalized using normalization factor generated by GeNorm using two reference genes *Ov-tubA* and *Ov-ubi*. The distribution is shown by cyan vertical boxes for acclimatization group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers. The differences between the groups resulted no significant by ANOVA analysis.

8.4 Discussion

The target genes of this study (*Ov-uch, Ov-stm, Ov-dat, Ov-TH, Ov-OP, Ov-CT*) are not subjected to transcriptional regulation throughout the sub-oesophageal mass (SUB) in response to social interaction. Analyzing the expression of target genes in the supra-oesophageal mass (SEM) the only gene that shows significant differences between control and trained animals is *Ov-stm*. This gene is up-regulated in response to social interaction suggesting a role in the regulation of animals' behaviour, as is the case in vertebrates (Martel *et al.*, 2007). In fact this gene is known to regulate the mechanisms of induction of LTP in response to innate fear (Shumyatsky *et al.*, 2005).

The genes *Ov-uch*, *Ov-stm*, *Ov-dat* and *Ov-th* are subjected to transcriptional regulation in response to social interaction in the optic lobes (OL).

It is interesting to note that all these genes, known to be involved in the processes of memory activated in response to learned fear in vertebrates, change their expression level also in response to innate fear in the optic lobes of octopus. Thus the outcome of this analysis suggests the potential involvement of these genes in the processes of memory storage that are known to be located in the optic lobes (Young, 1962,: Young, 1971).

It must be stressed however that while *Ov-uch* and *Ov-stm* show an increased expression, *Ov-dat* and *Ov-TH* are down-regulated in response to social interaction.

The response of the first two genes confirmed the hypothesis that they (*Ov-uch* and *Ov-stm*) may be involved in the octopus, as well as in vertebrates, in mechanisms of LTP and LTM formation triggered in this case in response to the innate fear and with the aim to set a behavioural response that can preserve the survival of the animal.

On the other hand, the decreased expression of second pair of genes (*Ov-dat* and *Ov-TH*) leads us to suggest that the abnormality and the forced social interaction for a solitary animal such as the octopus may result in a decrease of dopamine release mediated by the repression of gene expression involved in its synthesis in the dopaminergic districts.

CHAPTER 9

COMPARISON BETWEEN REAL-TIME qPCR EXPERIMENTS

At this point I think it is interesting to compare the outcomes of these two experiments with the aim to analyze how the expression of target genes in four different experimental conditions changes (Naïve, acclimatization, fear conditioning and social interaction). For RT qPCR, mRNA have been isolated from samples of the brain masses of different individuals. For fear conditioning experiment I pooled serial sections (see paragraph 7.2.2) and I extracted mRNA from these pools. For innate fear the whole masses were processed.
This difference may have affected the actual concentration of the mRNAs of the two 'sampling' techniques. However taking into consideration that the actual results are expressed in relation to the relative content of reference gene of each sample I assume this should not have affected the actual result.

9.1 Ubiquitin C-terminal hydrolase

O. vulgaris ubiquitin C-terminal hydrolase (*Ov-uch*) expression data are summarized in figure 9.1. Analyzing the *Ov-uch* expression levels in OL among four experimental groups (naïve, acclimatization, fear conditioning and social interaction) they seemed different and this observation was confirmed by ANOVA analysis and post hoc Bonferroni test ($F_{(3,43)} = 38.804$, $p < 0.0001$; naïve vs acclimatization $p < 0.0001$, naïve vs social $p < 0.0001$, acclimatization vs fear conditioning $p < 0.0001$, fear conditioning vs social $p < 0.0001$).
The ANOVA analysis conducted on the *Ov-uch* expression levels in the SEM of four experimental groups confirmed a different distribution of expression of this gene in each group of animals ($F_{(3,43)} = 26.606$; ; naïve vs acclimatization $p < 0.0001$, naïve vs social $p < 0.0001$, acclimatization vs fear conditioning $p < 0.0001$, fear conditioning vs social $p < 0.0001$).
In order to analyze the effect of behavioural experience on *Ov-uch* expression in the SUB, ANOVA analysis and post hoc tests have been conducted. The target gene expression in SUB was significantly changed by behavioural experience ($F_{(3,43)}$ =

44.442, $p < 0.0001$; naïve vs acclimatization $p < 0.0001$, naïve vs social $p < 0.0001$, acclimatization vs fear conditioning $p < 0.0001$, fear conditioning vs social $p < 0.0001$). Looking at the level of gene expression in different masses of CNS there is a high amount of *Ov-uch* mRNA in response to positive learning process studied in animals of both groups acclimatization and social interaction. We do not know if this could be due to differences between tasks or between the ITI used in trainings. Given that *uch* is one of the early genes involved in the mechanisms of formation, recall of long-term memory and even in reconsolidation I think more likely there may be an effect of the ITI. While the training used for fear conditioning is more easily comparable to a massed training because it involves the use of 1 minute ITI, the animals of both groups social interaction and acclimatization are subject to a spaced training because their training trials are spaced with 24 hours ITI. This could then generate a memory long lasting and probably require a more massive synaptic activation that would result in a higher level of activation of early genes.

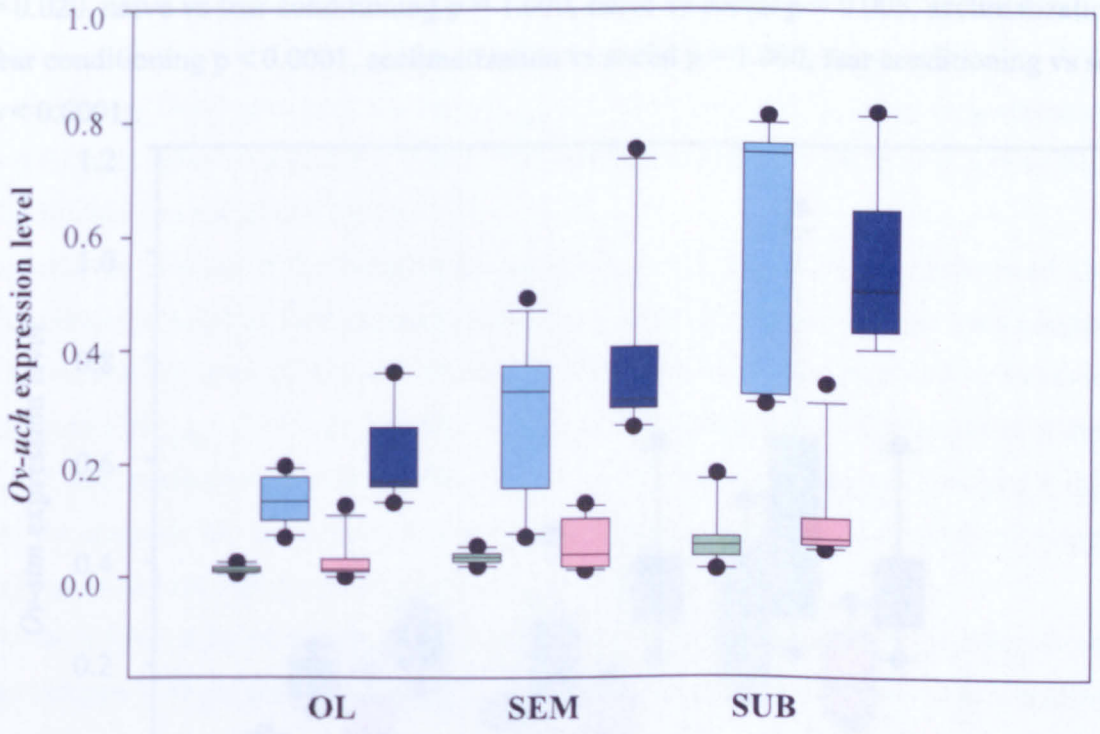


Figure 9.1: *ubiquitin C-terminal hydrolase* expression level in OL, SEM and SUB. The *Ov-uch* quantity has been normalized using two reference genes: *Ov-ubi* and *Ov-tubA*. The distribution is shown by green vertical boxes for naïve group, cyan boxes for acclimatization group, pink boxes for fear conditioning group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers.

9.2 Stathmin

O. vulgaris stathmin (*Ov-stm*) expression data are summarized in the figure 9.2. *Ov-stm* resulted differently expressed in each brain mass of animals belonging to four different experimental groups (naïve, acclimatization, fear conditioning and social interaction). ANOVA analysis and Bonferroni post hoc tests have been used to analyze the differences of the target gene expression in OL among the experimental groups ($F_{(3,43)} = 37.112$, $p < 0.0001$; naïve vs acclimatization $p < 0.0001$, naïve vs social $p < 0.0001$, acclimatization vs fear conditioning $p = 0.001$, fear conditioning vs social $p < 0.0001$).

Significant differences were found among *Ov-stm* expression levels in SEM of four experimental groups ($F_{(3,43)} = 18.852$, $p < 0.0001$; naïve vs acclimatization $p = 0.014$, naïve vs social $p < 0.0001$, acclimatization vs fear conditioning $p = 0.007$, fear conditioning vs social $p < 0.0001$).

Also in SUB some significant changes of *Ov-stm* expression levels were found in response to different behavioural experiences ($F_{(3,43)} = 12.608$, $p < 0.0001$; naïve vs acclimatization $p = 0.020$, naïve vs fear conditioning $p = 1.000$, naïve vs social $p = 0.005$, acclimatization vs fear conditioning $p < 0.0001$, acclimatization vs social $p = 1.000$, fear conditioning vs social $p < 0.0001$).

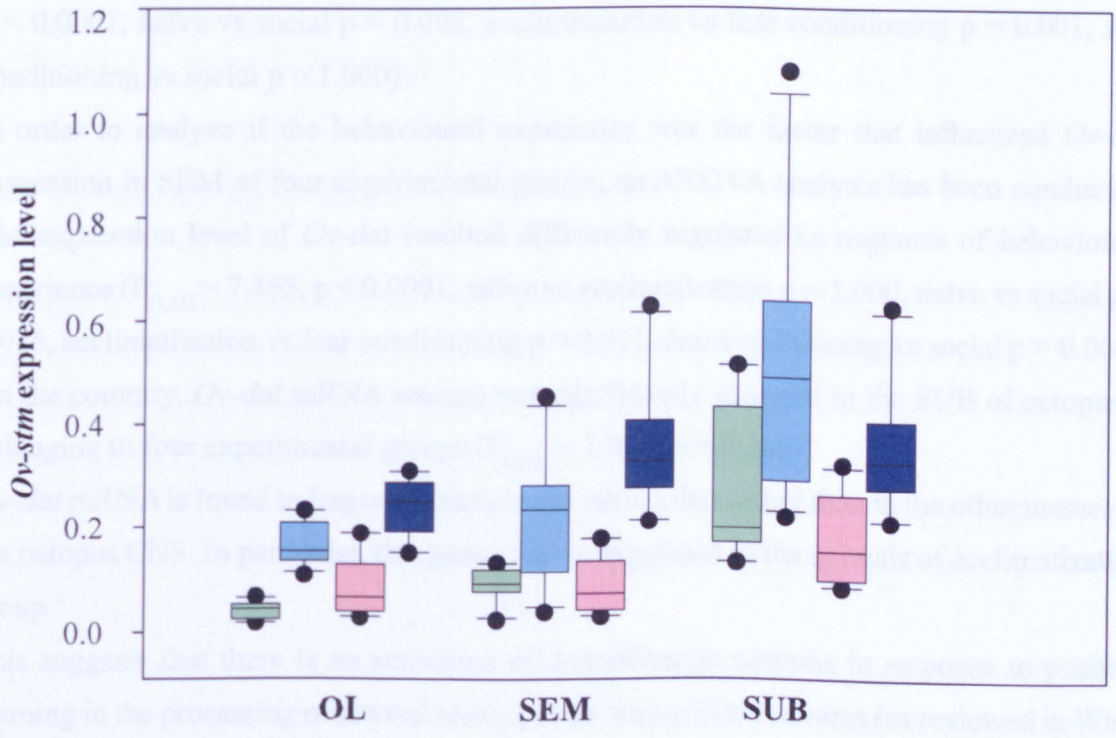


Figure 9.2 : *stathmin* expression level in OL, SEM and SUB. The *Ov-stm* quantity has been normalized using two reference genes: *Ov-ubi* and *Ov-tubA*. The distribution is shown by green vertical boxes for naïve group, cyan boxes for acclimatization group, pink boxes for fear conditioning group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers.

The octopuses of acclimatization and social interaction groups show a higher level of Ov-stm expression in OL and SEM in respect to the other groups. Instead the animals of acclimatization group show a higher amount of *Ov-stm* in the SUB.

It is also to note a high level of *Ov-stm* expression in SUB of naive animals. It is necessary to consider that *stathmin* is a gene known to be involved in the processes of innate and learned fear.

It can not exclude that the experience of capture may have resulted in the expression of innate fear and so caused the increase of *Ov-stm* expression in SUB of naive octopuses.

It remains to explain why this effect is measurable only in the SUB, where in addition there is a decrease in levels of *Ov-stm* in response to social interaction and learned fear in respect to the relative control groups.

9.3 Dopamine transporter

O. vulgaris dopamine transporter (*Ov-dat*) expression data were summarized in figure 9.3. *Ov-dat* expression level showed significant differences among OL of four experimental groups (naïve, acclimatization, fear conditioning and social interaction) as suggested by ANOVA analysis and Bonferroni post hoc test ($F_{(3,43)} = 15.674$, $p < 0.0001$; naïve vs acclimatization $p < 0.0001$, naïve vs social $p = 0.002$, acclimatization vs fear conditioning $p = 0.001$, fear conditioning vs social $p = 1.000$).

In order to analyze if the behavioural experience was the factor that influenced *Ov-dat* expression in SEM of four experimental groups, an ANOVA analysis has been conducted. The expression level of *Ov-dat* resulted differently regulated in response of behavioural experience ($F_{(3,43)} = 7.465$, $p < 0.0001$; naïve vs acclimatization $p = 1.000$, naïve vs social $p = 0.035$, acclimatization vs fear conditioning $p = 0.011$, fear conditioning vs social $p = 0.004$). On the contrary, *Ov-dat* mRNA amount not significantly changed in the SUB of octopuses belonging to four experimental groups ($F_{(3,43)} = 1.095$, $p = 0.362$).

Ov-dat mRNA is found in larger amounts in the optic lobes rather than in the other masses of the octopus CNS. In particular, this gene is more expressed in the animals of acclimatization group.

This suggests that there is an activation of dopaminergic neurons in response to positive learning in the processing of reward in octopus as occurs in vertebrates (as reviewed in Wise, 2006).

At the same time, however, also it is shown the request of dopamine in mediating the processes of consolidation activated in response to fear conditioning. It would suggest that even in the processes of aversive learning is required activation of dopamine neurons as observed in *Drosophila* in response to olfactory conditioning with electric shocks to punishing stimulus

(e.g. Schwaerzel *et al.*, 2003; Riemensperger *et al.*, 2005; Schroll *et al.*, 2006).

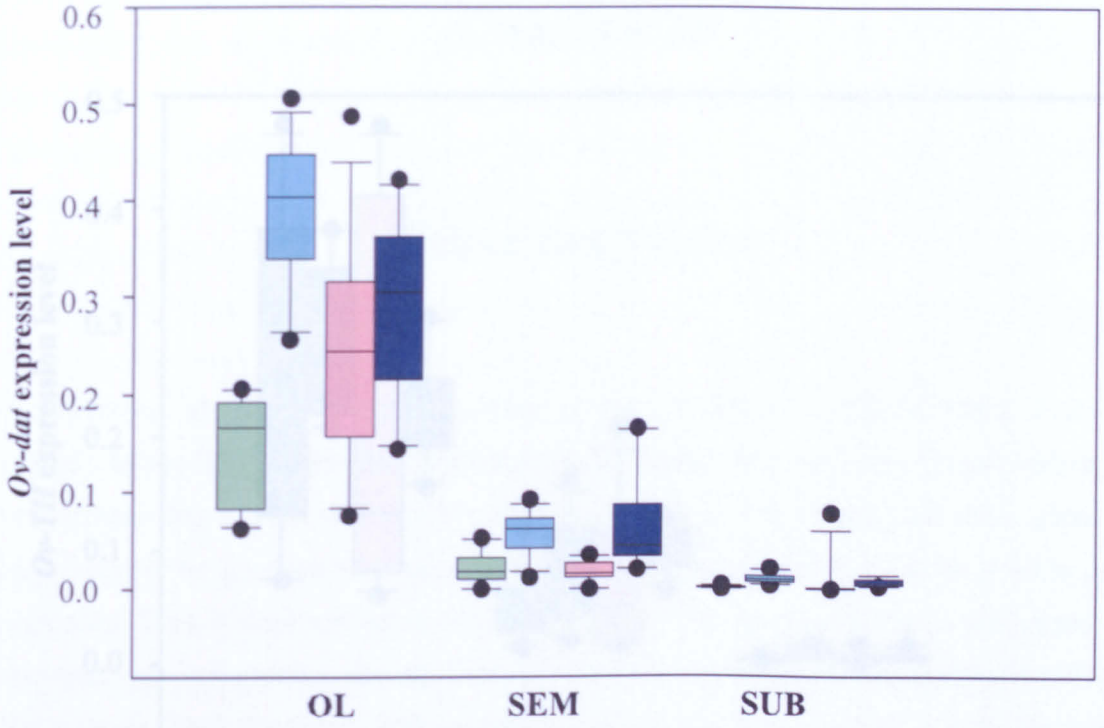


Figure 9.3 : *dopamine transporter* expression level in OL, SEM and SUB. The *Ov-dat* quantity has been normalized using two reference genes: *Ov-ubi* and *Ov-tubA*. The distribution is shown by green vertical boxes for naïve group, cyan boxes for acclimatization group, pink boxes for fear conditioning group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers.

9.4 Tyrosine hydroxylase

O. vulgaris Tyrosine Hydroxylase (*Ov-TH*) expression data are summarized in figure 9.4. Analyzing the *Ov-TH* expression levels in OL among four experimental groups (naïve, acclimatization, fear conditioning and social interaction) they seemed not different and this observation was confirmed by ANOVA analysis ($F_{(3, 43)} = 0.497$, $p = 0.686$).

The ANOVA analysis conducted on the *Ov-TH* expression levels in the SEM of four experimental groups confirmed the uniform distribution of expression of this gene in each experimental group ($F_{(3,43)} = 2.514$, $p = 0.072$).

In order to analyze the effect of behavioural experience on *Ov-TH* expression in the SUB, ANOVA analysis and post hoc tests have been conducted. The target gene expression in SUB was significantly changed by some behavioural experiences ($F_{(3,43)} = 6.302$, $p = 0.001$; naïve vs acclimatization $p = 0.001$, naïve vs fear conditioning $p = 0.641$, naïve vs social $p = 0.132$, acclimatization vs fear conditioning $p < 0.057$, acclimatization vs social $p = 0.519$, fear conditioning vs social $p = 1.000$).

As shown in Figure 9.4 *Ov-TH* is mainly expressed in the optic lobes as is the case in *Ov-dat*,

but there were no other (in addition to those previously commented) significant changes in expression between the different experimental groups studied.

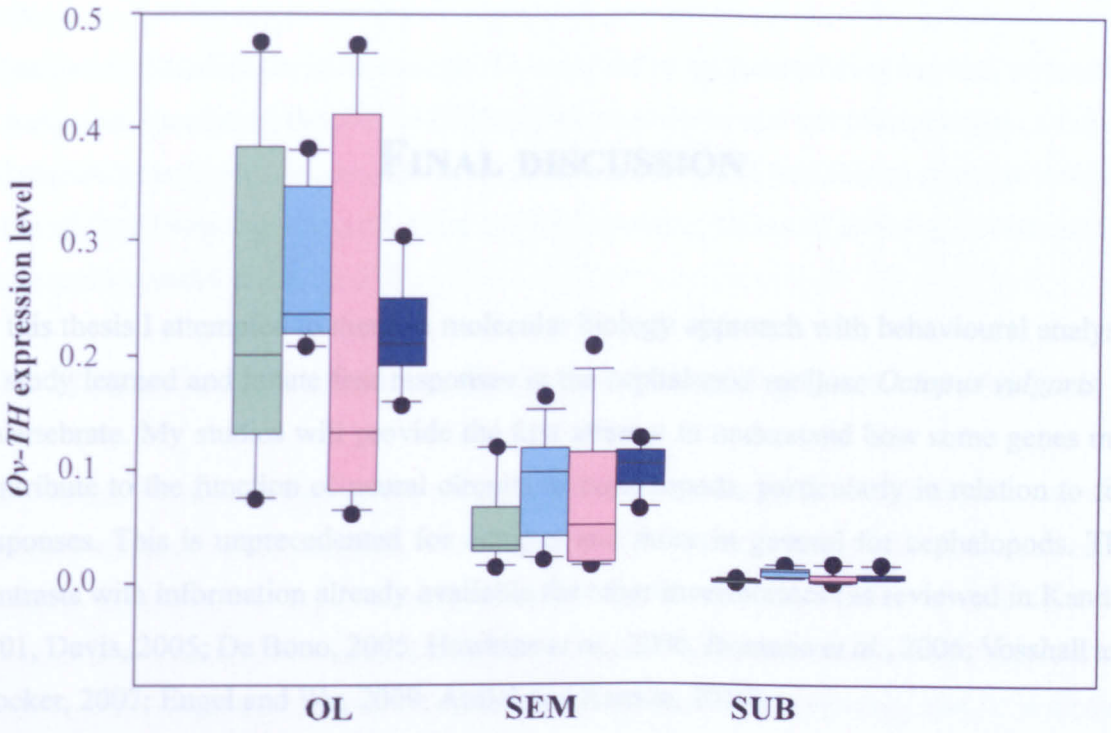


Figure 9.4 : *Tyrosine Hydroxylase* expression level in OL, SEM and SUB. The *Ov-dat* quantity has been normalized using two reference genes: *Ov-ubi* and *Ov-tubA*. The distribution is shown by green vertical boxes for naïve group, cyan boxes for acclimatization group, pink boxes for fear conditioning group and blue boxes for social interaction group, respectively. The box plot contains median (lines), 25th and 75th percentile (boxes) and 90th and 10th percentile (whiskers). Circles mark outliers.

CHAPTER 10

FINAL DISCUSSION

In this thesis I attempted to merge a molecular biology approach with behavioural analyses to study learned and innate fear responses in the cephalopod mollusc *Octopus vulgaris,* an invertebrate. My studies will provide the first attempt to understand how some genes may contribute to the function of neural circuits in cephalopods, particularly in relation to fear responses. This is unprecedented for octopus and more in general for cephalopods. This contrasts with information already available for other invertebrates (as reviewed in Kandel, 2001, Davis, 2005; De Bono, 2005; Hawkins *et al.*, 2006, Romano *et al.*, 2006; Vosshall and Stocker, 2007; Engel and Wu, 2009; Ardiel and Rankin, 2010).

This study may be divided in several steps:

1. design and tuning of behavioural protocols for the study of fear in the common octopus: conditioned (learned fear) and innate fear;
2. analysis of known cephalopods and octopus' gene sequences to search for potential target genes for this study and identification of novel ones;
3. study of the spatial expression of genes of interest in the brain of *O. vulgaris*;
4. analysis of the role played by CREB during consolidation and memory recall of conditioned fear;
5. analysis of the expression profiles of different genes in the brain of the octopus in response to learned and innate fear.

As mentioned, the *leit motif* of this Thesis is fear, in particular learned and innate fear. Learned fear was studied here using a novel fear conditioning training protocol, that revised an old one (Sanders and Barlow, 1971), using an artificial stimulus and an electric shock as a negative reinforcement. In addition, I studied innate fear by allowing octopuses, that are reported to be solitary in their lifestyle (Altman, 1967), to be forced to interact visually with conspecifics for even during feeding.

A genome sequencing for octopus is not avaliable; therefore I explored nucleotidic sequences available for cephalopods in GenBank and had access to two cDNA libraries (Ogura *et al.*, 2004; Brown and Fiorito, unpublished). This search has allowed me to identify sequences of genes: *α-tubulin*, *octopressin*, *cephalotocin*, *stathmin*; as for the other genes I started

appropriate experiments to obtain partial sequences. Once selected the target genes, I proceeded to study their expression in the nervous system of octopus by *in situ* hybridization experiments, this allowed to draw a preliminary map of their distribution in the individual lobes.

Subsequently I studied the involvement of each of the target genes during learning processes. In particular, I analyzed the role of CREB (phosphorylated and not phosphorylated forms) and studied gene expression by real-time qPCR experiments. I was able to compare baseline levels of "my" target genes with those induced by some forms of learning in response to innate and learned fear.

10.1 Behavioural studies: fear conditioning and innate fear

In my thesis, I described briefly the setting-up of a newly established training procedure of passive avoidance for *O. vulgaris*. This consists of: *i.* a short period of acclimatization to laboratory conditions, *ii.* a pre-training phase during which the animal is presented for the first time with an artificial stimulus (and the associated reward), *iii.* a training phase during which another artificial stimulus is presented but negatively reinforced, and *iv.* a testing phase (24 hours after training) during which the animal is presented with the same stimulus to assess memory recall in absence of any reinforcement.

During acclimatization I observed the complete recovery of the predatory capability of octopuses, predatory response that maybe affected by stress induced by capture and transfer from their natural environment to laboratory tanks.

During pre-training it was possible to observe the natural propensity of the animal to attack an artificial stimulus, never seen before, and the generalization of its predatory performance from natural to artifical stimuli. During training I measured octopuses ability to learn to avoid. After the initial attacks on the stimulus and following punishment, the animals propensity to attack appear reduced becoming more cautious in approaching the stimulus. The repeated administration of the shock, over trials, induced a change in the octopus' motor response which changed from full attack to approach, up-to withdrawing; as a consequence during trials the latency to attack the stimulus increased (see Borrelli, 2007). These responses can be easily compared to those of other invertebrates (e.g. *D. melanogaster*, *C. elegans*) or vertebrates (e.g. *M. musculus*). In fact, even those with the occurrence of danger they avoid it by escaping (i.e. withdrawing or avoidance) and by freezing depending on the situation. In case of man, for example, these behaviours are characterized also by facial and vocal communication signals that indicate the state of fear (as reviewed in Iliadi, 2009).

As LeDoux (1994) suggested, fear conditioning is an evolutionarily old mechanism for acquiring and storing information about harmful or potentially harmful stimuli and situations.

Behavioural expression of conditioned fear and its neural basis appear very similar in all species from fruit fly to human.

The behavioural protocol of avoidance described in this thesis allowed me to confirm the strong capabilities of the octopus to learn. Despite the limited size of the central nervous system and short life-span, animals can learn to avoid a stimulus even when it is very "attractive" (i.e. for octopus: artificial stimulus; honeybee: smell) or tasty (i.e. for fruit fly and honeybee: sucrose) and suddenly becomes a source of danger (i.e. for octopus, fruit fly and honeybee: electric shocks; for fruit fly: high temperatures, salt, quinine; for honeybee: toxin; for review see: Dukas, 2008; Wright *et al.*, 2010). As mentioned, the avoidance protocol I used with octopuses is based upon the study of Sanders and Barlow (1971; 1974); here however, I use an artificial stimulus instead of a natural one (i.e. crab).

The reason why I changed the kind of stimulus is due to the fact that the employment of a natural prey, as a stimulus to avoid, could have a negative effect on learning capability, by potentially changing motivational state; however to the best of my knowledge, a systematic study in this sense is missing. The risk is that administering a series of shocks to the animal may induce a generalized fear that affect the response to the stimulus and mask learning; this might occur in the cases of Barlow and Sanders (1971), but should be excluded in our conditions since all animals attacked the crab (i.e. natural stimulus) promptly at the end of the training to avoid the plastic ball.

The results I obtained with octopus, parallel those reported for other invertebrate species (e.g. for snail: Azami *et al.*, 2006; for honeybee: Wright *et al.*, 2010). Further study, will be necessary to compare the differences in memory recall after training with natural or artificial stimuli thus allowing an analysis of octopus' long-term memory for periods longer than those tested (24 h or 30 h; this study and Barlow and Sanders, 1971).

During a second series of behavioural experiments, I tested the influence that the variation of ITI could have on the learning rate and on memory recall. My results confirmed that changes in the ITI duration did not affect the learning performance as also suggested by Barlow and Sanders (1974). In addition, different ITIs did not affect memory recall in my experiments. These results seem to contrast with what has been demonstrated in several other species such as *Aplysia* (Botzer *et al.*, 1998), honeybee (Menzel *et al.*, 2001; Sandoz, *et al*, 1995), and the nematode (Beck & Rankin, 1997), where it has been shown that long ITIs or spaced training could produce longer memory than short ITIs or massed training procedures. I cannot exclude that the differences in the memory retention induced by variation of ITIs could not be appreciable at 24 hours after training in the octopus, but could be measurable only after a longer time. This occurs in the honeybee, where ITI variation have no effect upon animal performance when tested up to 3 h after training. However, 24 h after training, animals trained with 10 min ITI display a 'better' conditioned response (Sandoz *et al.*, 1995).

A similar response is also observed with moths; animals trained with different ITIs do not display different retention when tested at 15 min, but 120 min after training the animals trained with longer ITIs show higher level of retention than those trained with shorter ITI (Fans *et al.*, 1997).

Having found no effect due to the variation of the ITIs I also investigated the effect that the change in the number of trials as criterion could have on the performance of octopuses during the testing phase. As reported by Shomrat and co-workers (2008), octopuses trained using the 5-min ITI and criterion-4 (4 consecutive trials during which the octopus does not attack the stimulus) showed a lower level of retention compared to those trained with criterion-6. The same response was observed in animals trained using 1-min ITI and criterion-4. This suggests that there may be an effect on memory recall due to the number of trials of training. Similar results have been found by studying the response of moths in olfactory conditioning. Increasing the number of training trials from 2 to 10 led to more stable memories that lasts for a longer period (Fan et al., 1997). In the honeybee a good level of learning and memory can be observed after a single learning trial, but that this memory is more stable after multiple learning trials (Menzel, 1990; Menzel *et al.*, 1993). It remains still remarkable that for the octopus the change of the number of trials from criterion-6 to 4 (which in many cases led to a decrease of only 2 trials of the training) could have a significant effect on memory recall. In addition, during the testing phase there was an improvement of the animal's response to the presentation of the stimulus. In fact, the number of animals that attacked the stimulus decreased between the first and last testing trials. This suggests that the repeated presentation of unreinforced stimulus during the testing phase, i.e. 24 hours after training, may trigger a process of reconsolidation as occurs in other species (i.e. crab: Perez-Cuesta and Maldonado, 2009; rats: Inda *et al.*, 2011). It would certainly be interesting to conduct molecular and pharmacological experiments that can test the labilizazion and strengthening of memory.

Finally, I also tested the 'effects' of social interaction on octopuses' predatory response. Previous studies already showed the flexibility with which *O. vulgaris* responds to new environment (i.e. captivity) in which it has a limited space, a poor scenery and live prey that 'fall from sky'. Because of this 'plasticity', I decided to study their response to a strong change for a solitary animal: 'forced' visual interaction with conspecifics. In these experiments octopuses were in constant visual interaction with conspecifics and they were faced simultaneously to a live crab. The presence of a conspecific raised the level of competition, may lower the level of attention towards the prey, and could generate fear that could inhibit or delay the predatory response in one or both individuals (of the diade). As predicted, animals of the 'social' group showed significantly different predatory performances compared to the control group. This could suggest that the response of these animals was influenced by 'innate fear' a form of fear that is not the result of any learning process. During consecutive days,

conspecifics familiarize (even thought exclusively through continuous visual interaction; see Tricarico *et al.*, 2011). This familiarization induces a reduction of competition and/or a form of individual recognition between pairs of octopus (Tricarico *et al.*, 2011).

10.2 Cephalopods' and octopus' gene sequences

The search for genes to be tested for the aims of this thesis and the limited knowledge about the genome of cephalopods in general, prompted me to analyze nucleotide sequences available in GenBank for this taxon. I selected the sequences belonging to seven genera: *Octopus, Sepia, Loligo, Eledone, euprymna, Sepiola, Sepioteuthis.* This search produced 1814 nucleotide sequences, some of these were redundant and were removed from further analysis to improve and semplify downstream analysis. Among the 1814 sequences available more than 15% were redundant. I analyzed non redundant sequences by Blast2GO to help classifying their molecular function with the ultimate goal to identifying possible target genes for learning and memory studies. I also performed the same analysis on the nucleotidic sequences derived from cDNA libraries that are not available through GenBank (Gojobori's EST library; Brown's and Fiorito's cDNA library). Different functional categories have been identified in the dataset I had as available source of nucleotide sequences for octopus. Although several of them should be of interest(e.g. transport activity, regulation of transcription), only few have been considered suitable for the aim of this thesis: *α-tubulin*, *octopressin*, *cephalotocin* (from NCBI GenBank) and *stathmin* (from Gojobori's EST library). This was mainly due to focus the experiments, a strategy that seemed the most conservative way due to the necessary tuning-up of molecular biology experiments that are novel for octopus (and cephalopods).

By performing PCR on cDNA using degenerate or not degenerated primers I identified partial cDNA sequences for the *O. vulgaris* genes *TH* (1199 bp), *uch* (224 bp), *Stathmin* (871 bp) and *dat* (1113 bp). This approach was time consuming and allowed for the identification of a small number of new sequences, surely sufficient for this project, but not enough to significantly expand the limited knowledge of octopus DNA or RNA sequences. An RNA-seq approach could allow many advances in the characterization and quantification of octopus transcriptome (as reviewed in Ozsolak and Milo, 2011). A possible study of the transcriptome of the octopus central nervous system is a necessary step that has been used for other molluscs such as *Aplysia*; in this last case the identification of 65.000 non redundant nucleotidic sequences expressed in specific neurons or ganglia of CNS was very valuable (Moroz *et al.*, 2006).

The predicted amminoacid sequences of novel octopus cDNA sequences were aligned with the orthologous ones of other invertebrate and vertebrate species in order to measure

the percentage of identity. The identification of these genes is still a solid base for many interesting future studies. In this thesis, I studied their role in the learning and memory processes activated in response to innate and learned fear, but it could be equally interesting to know their involvement in response to other tasks (as reported in table 4.1). This approach opens the door to a new avenue for *O. vulgaris* as an 'animal model'. It makes possible the association of molecular genetic studies and behavioural analysis with the aim to identify genes and mechanisms involved in the complex behavioural processes. An approach that is already widely used in many other animal model (as reviewed in Kandel, 2001, Davis, 2005; De Bono, 2005; Hawkins *et al.*, 2006, Romano *et al.*, 2006; Vosshall and Stocker, 2007; Engel and Wu, 2009; Ardiel and Rankin, 2010).

In addition, some of the target genes of this thesis (i.e.*creb* and *uch*) can be regarded as key molecules for the consolidation and formation of LTM. These same molecules could be studied with the aim of increasing knowledge about the processes of reconsolidation and extinction (e.g. Mamiya *et al.*, 2009). Moreover, the identification of the remaining part of partial gene sequences could allow phylogenetic studies so limited in this animal model (i.e. Carlini *et al.*, 2000; Yokobori *et al.*, 2004).

10.3 The study of spatial expression of target genes in the octopus CNS

The *in situ* hybridization experiments I carried out provided preliminary information on the distribution of target and reference gene transcripts in the octopus brain.

Data obtained by these experiments are summarized in table 5.2 and indicate that these mRNAs are present in every brain mass SEM, SUB and OL. The distribution in each lobe is reported without quantitative information. Table 6.2 also reports the data on the discovery of the signal in the nuclear region of neurons or in the neuropil. The finding of *Ov-stm* and *Ov-ubi* mRNA in the neuropil supports the hypothesis that *de novo* protein synthesis may take place in axons and not only restricted to cell bodies (Wang *et al.*, 2010).

However, the ultimate goal of these experiments was to map the distribution of baseline expression of genes of interest in the various regions of the CNS of naive animals. This has allowed in the case of *dat* and *TH* to provide information on the distribution of noradrenergic and dopaminergic neurons a little more accurate than available so far (Messenger, 1996).

Moreover, the knowledge of the regions where the target genes are expressed in the octopus brain may eventually provide a way to find changes induced in response to fear experiences. The changes in response to fear conditioning have been found in many animal models. For example there was a significant increase of the expression of *creb* in the amygdala,

hippocampus and some thalamus nuclei, all parts of vertebrate lymbic system that mediates emotional response and is responsible for memory formation (as reviewed in Silva *et al.*, 1998; Josselyn and Nguyen, 2005). After fear conditioning training *TH* is up-regulated in the vertebrate brain regions that contriute to regulate learning, memory, attention and motor functions (e.g. Kobayashi and Sano, 2000). In addition, an increased expression of *stm* in the amygdala of rats in response to innate and learned fear has been reported (Shumyatsky *et al.*, 2005).

Finally, knowledge of parts of the brain where the genes of interest are expressed may even allow us to manipulate gene expression and observing the consequences on animal for studying gene function and the role played by this gene in a particular lobe of the CNS.

10.4 Fear conditioning in *O.vulgaris*: the role of CREB phosphorylation

Since very little is known about the ability of octopuses to recall information recorded during training and in relation with different forms of 'memory' (i.e STM, MTM, LTM) I approached the analysis of CREB phosphorylation after training and after the test with the aim to identify the role of this molecule in the octopus short- and long-term memory in response to fear conditioning. These preliminary results suggest that the mechanisms of short term memory (STM) were different from those of long term memory (LTM). The former did not induce a significant increase of phosphorylated CREB whereas LTM requires it, confirming the importance of CREB as key molecule for LTM formation also in *O. vulgaris*.

Early knowledge about CREB as a key molecule involved in the learning process originated from behavioural and molecular studies carried out on the marine snail, *Aplysia californica*. These studies indicated long-lasting increase of synaptic strength (i.e. Long-term Facilitation, LTF) and growth of new synaptic connections in a process that requires both mRNA and protein synthesis mediated by CREB activation (for review see Kandel, 2001). These and other studies on different invertebrate (i.e. *D. melanogaster*) and vertebrate species (i.e. *M. musculus*), proved the ground for establishing the role of CREB in the formation of long-term memory and in reconsolidation and extinction processes (Davis, 2005; Mamiya *et al.*, 2009; Radulovic & Tronson, 2010).

The results of the experiments I present on octopus, might suggest that I have not tested the involvement of CREB in LTM, but rather to have made an investigation on reconsolidation. Since the stimulus used during the testing phase was presented in five successive trials, thus there is the possibility that I recorded the ability of octopus to recall 'LTM' (in the first testing trail), but also an improvement of its performance between the first and fifth trial of tests. This would suggest that the repetition of the experience 24 hours after training and

even in absence of reinforcement may have improved the memory trace by a reconsolidation process that may strengthen LTM.

Therefore, it would be interesting to study the levels of CREB phosphorylation in response to a single test trial and/or to a prolonged stimulus presentation. In the first condition we would be able to test the role of CREB in LTM recall, but in the second case we will investigate its involvement in the process of extinction. It would also be interesting to carry out immunohistochemical experiments to detect if the changes in levels of phosphorylation in response to LTM, reconsolidation or extinction processes may affect different lobes within the CNS as occurs in vertebrates (Mamiya *et al.*, 2009).

10.5 Learned and innate fear: molecular analysis of circuitry

To study the relationship between learning processes and gene expression in octopus, I carried out real-time quantitative PCR experiments. In particular, I studied the changes of gene expression in response to fear conditioning able to induce learned fear and social interaction that could trigger innate fear. The control group for the first experiment was composed of naïve animals, while the octopuses of control group for innate fear were subjected to acclimatization in isolation. I studied the involvement of ubiquitin hydrolase, stathmin, tyrosine hydroxylase and dopamine transporter in both learned and innate fear, but for the last experiment I also studied the expression of two other genes: octopressin and cephalotocin. The analyses were conducted with the aim to study the changes of the target gene expression in the octopus brain masses (SEM, SUB, OL). However I also set up a method to study the gene expression in sub-regions of each mass. The experimental results are summarized in the table 10.1.

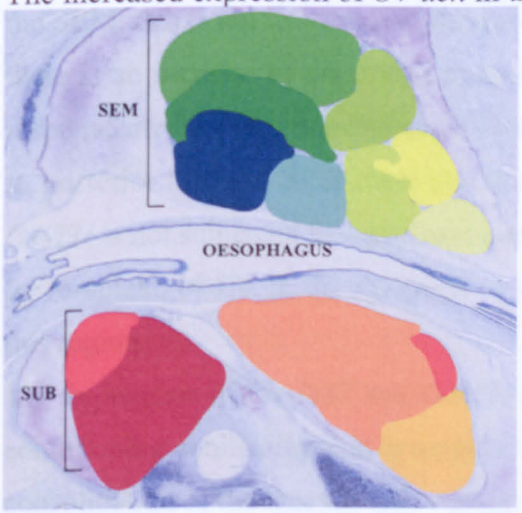
In response to learned fear (Naïve vs. Fear) I observed an increase of the expression of *Ov-uch*, *Ov-stm* and *Ov-TH* in the SEM or in some of its lobes. In the same mass *Ov-stm* was the sole to increase significantly as the consequence of the 'innate fear' protocol.

Ov-stm and *Ov-dat* decreased their expression in the SUB in response to fear conditioning, while no effect appears to be caused by social interaction. Finally, learned and innate fear protocols induced an increased expression of *Ov-stm* and *Ov-uch* in the optic lobes. Instead, *Ov-dat* and *Ov-TH* exibit an opposite pattern in response to fear conditioning and social interaction. In fact, in the first case, their level of expression increases and while in response to social interaction their expression is repressed.

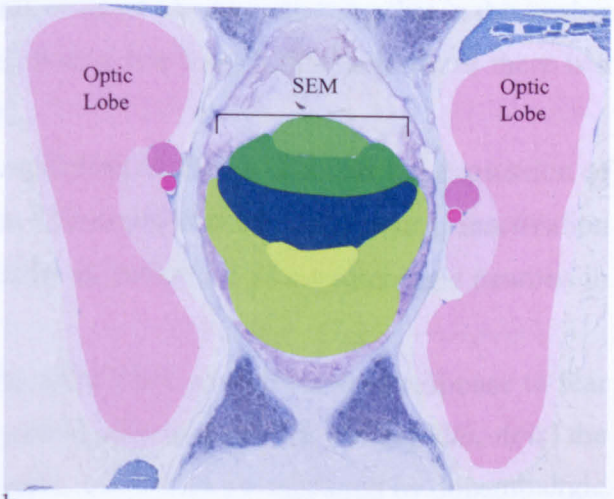
Table 10.1 - A tabularized overview of results of gene expression experiments. In this table I reported the increase(↑), decrease (↓) or the steady state of expression of each gene in the region of the brain considered, in response to learned (Naïve vs Fear) and innate fear (Accl vs Soc). A graphical summary of the distribution of genes in various lobes is provided in the sagittal (a) and horizontal (b) sections below.

Brain region	*Ov-dat*		*Ov-TH*		*Ov-stm*		*Ov-uch*		*Ov-CT*		*Ov-OP*	
	Naive vs Fear	Accl vs Soc	Naive vs Fear	Accl vs Soc	Naive vs Fear	Accl vs Soc	Naive vs Fear	Accl vs Soc	Naive vs Fear	Accl vs Soc	Naive vs Fear	Accl vs Soc
SEM	-	-	-	-	-	↑	-	-		-		-
Superior buccal lobe	-		↑		-		↑					
Posterior buccal lobe	-		↑		-		↑					
Inferior frontal lobe	-		↑		-		↑					
Sub-frontal lobe	-		↑		-		↑					
Superior frontal lobe	-		↑		-		↑					
Vertical lobe	-		-		↑		↑					
Sub-vertical lobe	-		-		↑		↑					
Anterior basal lobe	-		-		-		-					
Medial basal lobe	-		-		↑		↑					
Dorsal basal lobe	-		-		↑		↑					
SUB	-	-	-	-	↓	-	-	-		-		-
Prebrachial lobe	-		-		-		-					
Postbrachial lobe	-		-		-		-					
Anterior pedal lobe	-		-		↓		-					
Middle pedal lobe	-		-		↓		-					
Posterior pedal lobe	↓		-		↓		-					
Chromatophore lobe	-		-		-		-					
Magnocellular lobe	↓		-		↓		-					
Palliovisceral lobe	↓		-		↓		-					
OL	↑	↓	-	↓	↑	↑	-	↑		-		-
Optic gland	↑		-		↑		↑					
Olfactory lobe	↑		-		↑		↑					
Peduncle lobe	↑		↑		↑		↑					
Medulla	↑		↑		↑		↑					
Cortex	↑		↑		↑		↑					

The increased expression of *Ov-uch* in the lobes known as centers for learning and memory



a



b

storage confirms the involvement of this gene in octopus in the processes of synaptic plasticity, learning and long-term memory storage. This gene is in fact widely known as an early gene whose transcription is mediated by CREB. *Uch* codes for an enzyme that associates with the proteasome and increases its proteolytic activity. This regulated proteolysis is essential for long-term facilitation in *Aplysia*. The enhanced proteasome activity increases degradation of substrates that normally inhibit long-term facilitation. Thus, through induction of the hydrolase and the resulting up-regulation of the ubiquitin pathway, learning recruits a regulated form of proteolysis that removes inhibitory constraints on long-term memory storage in invertebrates (i.e. *Aplysia*, Hedge *et al.*, 1997) and in vertebrates (as reviewed in Di Antonio and Hike, 2004).

The increased expression of *Ov-stm* in SEM and in OL of octopuses subjected to innate and learned fear suggests that this gene plays a similar role in octopus to what is known in the vertebrate brain.

In fact it is known that amygdala enriched *stathmin* is required for the expression of innate fear and the formation of memory for learned fear. Stathmin is a cytosolic phosphoprotein involved in microtubular dynamics by regulating both the formation of microtubules and their disassembly. It has been found that its expression in the neural circuitry of fear in the adult mouse brain is essential for amygdala long-term potentiation (LTP), fear behaviour and social interaction (Shumyatsky et al 2005; Martel et al., 2008).

Interestingly, *Ov-stm* undergoes a negative regulation in response to fear conditioning in the SUB. One could hypothesize that this decrease in the expression of *stathmin* may be required to promote the dendritic arborization as observed in cultured Purkinje cells (as reviewed in Conde and Càceres, 2009). This suggests that the synaptic architecture is able to change and that these changes could be related to variations in microtubular dynamics. Surely this is a hypothesis that requires further studies to understand the relationship between microtubule dynamic, synapse formation, plasticity of neurons in the octopus.

The fear conditioning promotes the increased expression of *Ov-TH* in the anterior lobes of the SEM, this is not followed by increased activation of *Ov-dat* indicating that in this region there is an increase of dopamine metabolism with a low inactivation activity or there is a response mediated by noradrenergic neurons.

In posterior lobes of SUB there is a down-regulation of the *Ov-dat*, but the expression of *Ov-TH* is not subjected to significant changes. This could indicate a decrease of inactivation by re-uptake of DA in the synapses or probably an activation of noradrenergic neurons in this region.

The expression of *Ov-DAT* and *Ov-TH* in the optic lobes are increased in response to fear conditioning confirming that in octopus as in other invertebrates (i.e. *Drosophila*, *Apis*) the consolidation of the learned task with an aversive reinforcers are mediated by dopaminergic

pathways (e.g. Schwaerzel *et al.*, 2003; Riemensperger *et al.*, 2005; Schroll *et al.*, 2006).
In the optic lobes of animals subjected to social interaction there are decreased levels of expression of these two genes is likely to indicate that the normal process of acclimatization (positive learning) associated with a reward is mediated by processes that involve dopaminergic neurons, but social interaction blocks the request of dopamine down-regulating both *Ov- dat* and *Ov-TH*. Thus social interaction probably activates a process mediated by other neurotransmitters such as serotonin or octopamine.
As it can be seen in the table no change was observed about the *Ov-CT* and *Ov-OP* in response to social interaction. This result may be unusual given the known involvement of the orthologous of these genes (i.e. Oxytocin and vasopressin) in social memory and attachment, sexual and maternal behaviour, social recognition and aggression in vertebrates (as reviewed in Caldwell *et al.*, 2008; Lee *et al.*, 2009). Before reaching hasty conclusions, however, I think it is necessary to consider that the formation of a social memory of individuals is therefore vital, and in rodents relies primarily on volatile and pheremonal olfactory cues. In our experimental condition the interaction of octopuses was only a visual interaction, it is not possible any chemo-tactile experiences which probably could activate *Ov-CT* e *Ov-OP* and mediate the social memory formation and the individual recognition (Tricarico et al., 2011).

10.5.1 Comparison between real-time qPCR experiments

At this point I think it is interesting to compare the outcomes of these two experiments with the aim to analyze how the expression of target genes changes in four different experimental conditions (Naïve, acclimatization, fear conditioning and social interaction).
I observed an increase of *Ov-uch* mRNA in response to positive learning process; a condition that resulted to occur in octopuses during acclimatization and social interaction. Many reports refer to *uch* as one of the most important early genes involved in the learning processes. It is interesting to note that the different behavioural protocols utilized in this thesis make a different 'use' of ITI having fear conditioning very short intervals comparable to massed training, while social interaction and acclimatization are more easily treatable as spaced presentations (trials are spaced by 24 hours). Future experiments are needed to address this issue in a systematic way, since spaced presentations may induce massive synaptic activation that would result in a higher level of activation of early genes. Other factors cannot be excluded, but all remain to be evaluated.
As mentioned several times in this thesis *stathmin* is a gene known to be involved in fear. My results suggest that *Ov-stm* is expressed with higher levels in OL and SEM in animals exposed to crabs for several days either in isolation or in 'social' conditions. Levels of the mRNA of this gene are elevated in the SUB in animals not exposed to a particular training

protocol (*sensu lato*). I have no valid interpretation for this result; considering the fact that the SUB is essentially considered to be a center of motor control and coordination without apparent role in associative or any other forms of plasticity. Therefore it remains to be explored what the role of *Ov-stm* may be in brain processing and function.

It can not be excluded that the experience of capture may have resulted in the expression of innate fear and so caused the increase of *Ov-stm* expression in SUB of naive octopuses, but the question wheter this is so restricted to a not-associative area remains unclear.

My results indicate that there is an activation of dopaminergic neurons in response to positive learning and reward in the octopus as occurs in vertebrates (as reviewed in Wise, 2006). In addition dopamine related genes seem involved in fear conditioning. This lead to hypothesize that aversive learning in *O. vulgaris* requires activation of dopamine neurons as observed in *Drosophila* in response to olfactory conditioning (e.g. Schwaerzel *et al.*, 2003; Riemensperger *et al.*, 2005; Schroll *et al.*, 2006).

10.6 Conclusion and future directions

The aim of my PhD was to contribute to the knowledge of the molecular mechanisms underlying learning and memory processes in the Cephalopod Mollusc *Octopus vulgaris*. I found the sequences of some target genes and set-up a series of experimental tools and approaches that may help in increasing the use of *O. vulgaris* in the analysis of the biological machinery underlying learning and memory and more in general of behavioural plasticity. To the best of my knowledge, such an analysis is unprecedented for Cephalopod species.

These experimental approaches helped me to understand some of mechanisms activated in response to learned and innate fear in the octopus brain, but many other successive studies will be necessary to identify the molecular mechanisms which leads the complex behavioural responses of this intelligent and fascinating animal. My results contribute to the understanding of the involvement of CREB in the processes of memory formation and recall, of the role played by *Ov-stm* and *Ov-uch* in the processes activated in response to learned and innate fear, and of the function of *Ov-dat* and *Ov-TH* in processing of information coming from training with positive (i.e. acclimatization) and negative (i.e. fear conditioning) reinforcements. Moreover, octopus' brain regions where these genes are expressed and change their expression on the basis of behavioural experiences have been proposed. It would be interesting to identify more specifically the neural circuits involved in these processes to understand how these generate outputs able to regulate learning and memory processes.

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